Dietary Determinants of Age-Related Macular Degeneration

DIETARY DETERMINANTS OF AGE-RELATED MACULAR DEGENERATION

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ABSTRACT

Age-related macular degeneration (AMD) is the most common cause of irreversible blindness in older Americans. There has been a long standing interest in the role of diet in the development of AMD. As early as the first National Health and Nutrition Examination Survey in the 1970s, higher intakes of fruits and vegetables were inversely correlated with the prevalence of AMD. Carotenoids and omega3 fatty acids are the most studied dietary factors due to strong biological plausibility. However, evidence from epidemiologic studies and clinical trials on the relations has been inconsistent.

Chapter I prospectively examined the intakes of lutein/zeaxanthin and other common carotenoids in relation to the risk of AMD over more than two decades of follow-up among two large US cohorts, the Nurses' Health Study and Health Professionals Follow-Up Study. We assessed nutrient intakes by repeated food frequency questionnaires. We also computed bioavailable plasma carotenoid scores directly from food intake using validated regression models. Cox proportional hazards models were used to compute the associations. Higher intakes of bioavailable carotenoids (except lycopene) were inversely associated with advanced AMD but not intermediate AMD. Analyses based on bioavailable intakes resulted in stronger associations than conventional nutrient intakes.

Chapter II prospectively evaluated the marine long-chain omega3 fatty acids. We found that long-chain omega3 fatty acids were inversely associated with visually significant

intermediate AMD. There was no association with advanced AMD; however, the totality of current evidence for advanced AMD is also discordant.

Chapter III further investigated the plant-derived omega3 fatty acids, α-linolenic acid (ALA). We found that higher intake of ALA was associated with intermediate AMD before 2002 but not after. This coincides with the same time period when *trans* ALA was found in our participants' blood and in mayonnaise, a primary food source of ALA. Whether *trans* ALA mediates this positive association warrants further studies.

Although randomized trials are usually believed as the "gold standard", dietary factors are hard to be adequately studied by randomized trials due to the complexities of diet and disease relations. Thus, findings in this thesis from large long-term prospective cohort studies provide the next best form of evidence.

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CHAPTER I

INTAKES OF LUTEIN/ZEAXANTHIN AND OTHER CAROTENOIDS AND AGE-RELATED MACULAR DEGENERATION

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ABSTRACT

Importance: Despite strong biologic plausibility, evidence from epidemiologic studies and clinical trials on the relations between intakes of lutein and zeaxanthin and age-related macular degeneration (AMD) has been inconsistent. The roles of other carotenoids are less thoroughly investigated.

Objective: To investigate the associations between intakes of carotenoids and AMD.

Design: Prospective cohort study.

Setting: Nurses' Health Study and Health Professionals Follow-Up Study.

Participants: 63,443 women and 38,603 men were followed from 1984 and 1986, respectively, until 2010. All participants were 50 years and older, and free of diagnosed AMD, diabetes, cardiovascular disease and cancer at baseline.

Exposure: We assessed food intake by repeated food frequency questionnaires at baseline and follow-up. We computed predicted plasma carotenoid scores directly from food intake using validated regression models to account for bioavailability and reporting validity of different foods.

Main Outcome and Measure: We confirmed 1,361 incident intermediate and 1,118 advanced AMD cases (primarily neovascular AMD) with a visual acuity of 20/30 or worse by medical record review.

Results: In analyses based on a predicted plasma lutein/zeaxanthin score, we found a risk reduction for advanced AMD of about 40% in both women and men (pooled hazard ratio comparing extreme quintiles = 0.59, 95% CI = 0.48 - 0.73; p for trend, <0.001). Other predicted

plasma carotenoid scores, including β-cryptoxanthin, α-carotene and β-carotene, were associated with a 25% to 35% lower risk of advanced AMD. The hazard ratio comparing extreme quintiles for the predicted plasma total carotenoid index was 0.65 (95% CI = 0.53 - 0.80; p for trend, <0.001). We did not identify any associations of carotenoids, either as predicted plasma score or calculated intake, with intermediate AMD.

Conclusion: Higher intake of bioavailable lutein/zeaxanthin is associated with a long-term reduced risk of advanced AMD. Given that some other carotenoids are also associated with a lower risk, a public health strategy aimed at increasing dietary consumption of a wide variety of fruits and vegetables rich in carotenoids may reduce the incidence of advanced AMD.

I. Introduction

Age-related macular degeneration (AMD) is the leading cause of blindness in the developed world,[1,2] affecting 6.5% of persons aged 40 years and older in the US, with 0.8% afflicted by advanced AMD.[3] The prevalence of AMD prevalence is projected to increase by 50% in the next couple of decades[4-6] as a consequence of exponential population aging, the lack of a cure or any effective means of primary prevention other than smoking cessation.[7]

Carotenoids are fat-soluble plant pigments found in red, yellow, orange and dark-green fruits and vegetables. Of more than 600 carotenoids, 6 are commonly found in the human diet and serum: lutein, zeaxanthin, α-carotene, β-carotene, lycopene and β-cryptoxanthin.[8] Lutein and zeaxanthin are selectively concentrated in the macula,[9,10] where they are hypothesized to protect against AMD by absorbing blue light, quenching free radicals and stabilizing cell membranes.[11] However, in spite of compelling biologic plausibility, epidemiologic studies have not yielded consistent findings[12-15] and long-term, well-powered prospective cohort studies are lacking. The recently concluded Age-Related Eye Disease Study 2 (AREDS 2) trial was unable to confidently demonstrate protective effects of lutein/zeaxanthin[16,17] and whether lutein/zeaxanthin may protect against early AMD also remains unknown. Some other carotenoids, such as α-carotene, β-carotene and lycopene, found in the retinal pigment epithelium (RPE) and choroid,[18] have been inconsistently linked to a lower risk of AMD.[12-15,19,20]

We previously reported a suggestive inverse association of lutein/zeaxanthin with advanced AMD[21] and some associations for other carotenoids.[22] With an additional decade of follow-up and the occurrence of a large number of additional incident AMD cases, we aimed to provide more detailed insights into the roles of carotenoids in the development of AMD.

II. Methods

Study Population

The Nurses' Health Study (NHS) is an ongoing prospective cohort initiated in 1976 that includes 121,700 US female registered nurses aged 30 to 55 years at baseline. The Health Professionals Follow-up Study (HPFS) was initiated in 1986 and includes 51,529 US male health professionals aged 40 to 75 years at baseline. Both cohorts are predominantly white (NHS: >98%; HPFS: >91%), and have high rates of long-term follow-up (>95%). The study protocol was approved by the Institutional Review Boards at the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health.

We restricted the study population to participants ≥50 years, and censored participants at age 90 to alleviate concerns of low reporting validity (NHS, n=0; HPFS, n=526). At baseline, we excluded participants who did not return the initial FFQ, left the entire fruit and vegetable sections blank or had more than 70 food items blank, reported implausible dietary intake (<500 or >3500 Kcal/d for the NHS and <800 or > 4200 Kcal/d for the HPFS) (dietary exclusions, NHS, n=46,142; HPFS, n=1,647), had prevalent AMD, cancer (except nonmelanoma skin cancer), diabetes or cardiovascular disease (disease exclusions, NHS, n=8,536; HPFS, n=5,709). To minimize detection bias, we also excluded participants who never reported an eye exam over follow-up (NHS, n=3,362; HPFS, n=4,763) and excluded from analysis the person-time during any two-year interval in which a participant did not report an eye exam. In sensitivity analyses including intervals lacking an eye exam results did not materially change. Participants contributed person-time to the analysis from the return of baseline questionnaire or reaching 50 years old to the confirmed diagnosis of AMD, death, loss to follow-up or the end of follow-up (05/31/2010 for the NHS and 01/31/2010 for the HPFS), whichever occurred first. By 2010, a

total of 63,443 women and 38,603 men contributed to the analysis.

AMD Ascertainment

Our case definition has been previously validated.[23] When a participant reported a diagnosis of AMD on a biennial questionnaire, we requested informed consent and then contacted the participant's eye doctor to confirm the diagnosis by review of medical records. We excluded cases with only small hard drusen (< 63μm diameter). We defined intermediate AMD as having at least one of the following signs: intermediate drusen (≥ 63 and <125 μm), pigment abnormalities, large drusen (≥ 125 μm) or any non-central geographic atrophy (GA). We defined a subgroup of intermediate AMD as having at least one large druse or any non-central GA, the most likely to progress to advanced AMD.[24] We defined neovascular AMD as having any of the following: RPE detachment, subretinal neovascular membrane, disciform scar, or history of treatment with laser, photodynamic, or anti-VEGF therapy for AMD. Central GA was defined as having a central geographic atrophy lesion involving the center of the macula. Advanced AMD included neovascular AMD and central GA. Additionally, all our case definitions included a visual acuity of 20/30 or worse due primarily to AMD except for those neovascular AMD cases that had anti-VEGF therapy. The person was used as the unit of analysis, and the worst eye was used for classification.

In 2010, we recontacted approximately 2,300 previous AMD patients in the NHS who had AMD signs (either met or unmet our case definitions at that time) to ascertain whether or not their AMD may have progressed. Those cases accrued by recontact questionnaires were not included in the main analysis due to evidence of reverse causation bias (e.g. patients increased dietary intakes of carotenoids after the initial diagnosis of AMD; see **Results**).

Dietary Assessment of Carotenoids

We began follow up in 1984 for the NHS and 1986 for the HPFS, when the first comprehensive FFQ with an expanded section on fruit and vegetable intake was administered, and updated dietary intake every four years. On the FFQs, commonly used units or portion sizes (e.g., 1 orange or ½ cup broccoli) are specified for each item. The FFQs contained at least 15 questions for fruit and juice intake and 30 questions for vegetable intake. Participants were asked to report how often, on average over the past year, they had consumed each food item (responses ranging from "≤1 time per month" to "≥6 times/day"). Use and dosage of β-carotene and multivitamin supplements was assessed by biennial questionnaires with additional information on brands for multivitamins, whereas lycopene supplements were only inquired about from 2002 onwards. We calculated nutrient intakes by multiplying the consumption frequency of each food by the nutrient content of the specified food portion summing across all foods. Nutrient values were energy-adjusted using the residual method.[25] The FFQ has been validated in both cohorts and had good reproducibility and validity in measuring a wide range of foods and nutrients.[26-30]

Because of variation in assessment validity and bioavailability across carotenoid-containing foods, calculated intakes of carotenoids from FFQs may not adequately represent the more biologically relevant internal dosage. We thus utilized a previously validated empirical prediction model among 4,180 nonsmoking women in the NHS that related carotenoid-containing foods directly to the measured plasma carotenoid level using linear regression.[31] We made two changes to the original prediction model: 1) we excluded cucumber from the prediction model of β-cryptoxanthin because it was only asked in the 1986 FFQ; 2) we further developed a prediction model for food-sourced β-carotene by excluding β-carotene supplement users in 1990. The final prediction models used in this paper were shown in Supplementary

Table 1.1. The regression coefficient of each food in the model reflected a weighted contribution to the bioavailable level. We then derived predicted plasma carotenoid scores for all participants by multiplying the consumption frequency of each food by its regression coefficient and summing across all foods. We created a total carotenoid index by first categorizing each predicted plasma score into quintiles and then summing the quintile scores across all carotenoids yielding a final score ranging from 5 to 25. The empirical prediction model demonstrated improved assessment of carotenoid intakes compared to conventional food composition based method.[31]

Statistical Analysis

Main Analysis

We calculated the cumulative average for predicted plasma carotenoid scores by averaging scores from all available FFQs up to the start of each two-year risk interval. We used time-varying multivariate Cox proportional hazards model to estimate the hazard ratios (HR) and 95% confidence intervals controlling for known and suspected risk factors. We assessed the linear trend across categories by modeling the median level of each category as a continuous variable. We examined the possible non-linear relations between carotenoids and AMD non-parametrically by the likelihood ratio test comparing a model with only the linear term to a model with the linear and restricted cubic splines with four knots.[32]

To assess whether the associations between carotenoids and AMD would vary by prespecified risk factors including age, BMI, smoking status, and postmenopausal hormone use, we created interaction terms between carotenoids and these variables and tested their significance using likelihood ratio tests. In exploratory analysis, we investigated the independent association of

each carotenoid adjusting for all other carotenoids as a composite variable (sum of the quintile score of each carotenoid).

We performed the analyses separately in each cohort and pooled the results with an inverse variance-weighted meta-analysis using the fixed-effects model. We used an α- level of 0.05 without adjustment for multiple comparisons. SAS was used to perform the analyses (SAS 9.3, SAS Institute, Cary, NC).

Recontact Analysis in the NHS

To assess whether among recontacted patients an initial diagnosis of AMD was associated with change in dietary intakes of carotenoids, we followed the method that has been outlined in the study by Bernstein et al.[33] Briefly, we fit a generalized linear model accounting for within-person repeated dietary measures as below.

Change in calorie-adjusted carotenoid intake = β_1 (time interval) + β_2 (AMD) + β_3 (time interval X AMD) + β_4 (total calories)

Change in calorie-adjusted carotenoid intake = for example, change in calorie-adjusted carotenoid intake in 1986 was the calorie-adjusted intake in 1984 subtracted from the calorie-adjusted intake in 1990

Time interval=Time interval-7, centered at the 7th interval for model interpretability; 13 intervals ranged from 1984 to 2010

β_1= Change in calorie-adjusted carotenoid intake per 1 interval increase in non AMD participants

β_2= Change in calorie-adjusted carotenoid intake associated with a diagnosis of AMD during the middle of follow-up

β_3= Difference in the rate of change in calorie-adjusted carotenoid intake comparing AMD patients to non-AMD participants (*of the most interest*)

We compared the results from main follow-up with the results from main follow-up plus recontact to assess the degree of reverse causation bias. To control for this bias in the analysis including recontacted cases, we first attempted stopping updating the patient's diet after the first diagnosis of AMD and carried forward the diet before the diagnosis throughout the rest of follow-up. However, because stop updating diet may lead to misclassification especially when there is a temporal trend in carotenoid intake, we created z-scores of carotenoid intake during each follow-up cycle by subtracting the population's mean carotenoid intake from each participant's own intake and then divided by the population mean's standard deviation. We stopped updating a participant's z-score upon the first diagnosis of AMD.

III. Results

Main Analysis

During 26 years of follow-up in the NHS and 24 years in the HPFS, we confirmed 1,361 incident intermediate and 1,118 advanced AMD cases (>96% neovascular AMD). The median age of AMD onset was 73 years in women and 76 years in men.

In 1996 (the middle of follow-up), participants at the highest cumulative average predicted plasma score of lutein/zeaxanthin were likely to be more physically active, smoke less, consume more fruits and vegetables and score higher in aHEI. They also had higher calculated intakes of lutein/zeaxanthin and other carotenoids (Table 1.1).

Predicted plasma carotenoid scores were strongly correlated with their respective calculated intakes (Spearman correlation, r = 0.67 to 0.90), and with each other (e.g., r=0.64 between

lutein/zeaxanthin and food-sourced β-carotene, r=0.67 between α-carotene and food-sourced β-carotene). Lycopene had the weakest correlations with all other carotenoids ($r \leq 0.18$).

We identified an inverse association with advanced AMD for predicted plasma carotenoid scores of: lutein/zeaxanthin, β-cryptoxanthin, α-carotene, food-sourced β-carotene, total carotene and total carotenoid index (Table 1.2). Predicted plasma lutein/zeaxanthin score and total carotenoid index had a linear relationship with advanced AMD within the range of dietary intake (Figure 1.1). Carotenoids other than lycopene had a similar linear relation (all p for linearity < 0.05; all p for non-linearity > 0.10; graphs not shown). For the outcome of intermediate AMD, we did not observe any association for any predicted plasma carotenoid scores (Table 1.2). The results did not materially change when restricted to a subgroup of intermediate AMD with large drusen or non-central GA (283 in the NHS and 80 in the HPFS; data not shown).

Because AREDS2 raised the concern for the competitive absorption between lutein/zeaxanthin and β-carotene, in a sensitivity analysis we excluded β-carotene supplement users (4.5% of the person-years in the NHS and 10% in the HPFS) from the analysis of predicted plasma lutein/zeaxanthin score. However, neither the HR for advanced AMD (HR=0.58; 95% confidence interval (CI): 0.47 – 0.72; p for trend, < 0.001) nor the HR for intermediate AMD (HR=0.90; 95% CI: 0.75 – 1.09; p for trend, 0.26) was essentially altered.

In secondary analyses using calculated intakes of carotenoids, lutein/zeaxanthin, β-cryptoxanthin, α-carotene and food-sourced β-carotene were also inversely related to advanced AMD in the NHS (Supplementary Table 1.2); However, none of them was associated with advanced AMD in the HPFS (Supplementary Table 1.3).

The primary food sources for measured plasma carotenoid levels in the NHS[31] were cooked and raw spinach for lutein/zeaxanthin, oranges and orange juice for β-cryptoxanthin, cooked and raw carrots for both α- and β-carotene, and tomato sauce for lycopene (Figure 1.2), consistent with NHANES.[34] These foods were generally inversely related to advanced AMD, although with variation for specific forms of these foods (Figure 1.2). The inverse association between tomato sauce and advanced AMD was primarily attributed to that in the HPFS (HPFS, HR comparing ≥ 2 servings/wk to almost never = 0.60; 95% CI = 0.39 – 0.93; p for trend, 0.01). Cooked spinach and orange juice had an inverse association with intermediate AMD. Although not the primary source of α-carotene, bananas, which predicted plasma α-carotene level in our sample, was inversely relate to intermediate AMD (HR comparing ≥ 5 pieces/wk to almost never = 0.83; 95% CI= 0.70-1.00; p for trend, 0.003).

In an exploratory analysis adjusted for all other carotenoids as a composite score, only lutein/zeaxanthin and α-carotene persisted with an inverse association with advanced AMD (Figure 1.3). β-cryptoxanthin, β-carotene and lycopene collectively did not have an inverse association after accounting for lutein/zeaxanthin and α-carotene; nor did β-cryptoxanthin and β-carotene combined after accounting for all other carotenoids. In the sensitivity analysis in which we entered each individual carotenoid in the same model, the inverse association for lutein/zeaxanthin and α-carotene persisted (lutein/zeaxanthin, HR= 0.66, 95% CI= 0.50 to 0.87; α-carotene, HR= 0.75, 95% CI= 0.59 to 0.96).

Plasma carotenoid scores except lycopene seemed to be associated with a lower risk of total AMD among postmenopausal women currently using exogenous hormones compared to those not currently using (Supplementary Figure 1.1).We also found a suggestive stronger inverse association for all predicted plasma carotenoid scores except lycopene with advanced AMD in

those 75 years and older and this was most pronounced for lutein/zeaxanthin (p for interaction, 0.04) (Supplementary Figure 1.2 A). We found similar HRs in never compared to ever smokers (all p for interaction > 0.25) (Supplementary Figure 1.2 B).

Recontact Analysis in the NHS

Among all those who had responded to our recontact questionnaires, 558 patients met our case definition including 300 intermediate and 258 advanced AMD cases. Among them, 422 patients' AMD status progressed after the first diagnosis; the median time to AMD progression was 5.7 years (IQR: 1.7 – 10.3). From the date of initial diagnosis to the date of progression, lutein/zeaxanthin intake has significantly increased compared to those who did not have AMD during the same period (Supplementary Table 1.4). When stratified by the case status at the time of recontact, advanced AMD patients increased the intake of lutein/zeaxanthin more than intermediate AMD patients. There were no significant changes of intakes for other carotenoids.

After adding recontacted cases, 1,043 intermediate and1,002 advanced AMD cases were included in the analysis in comparison with 983 intermediate and 773 advanced AMD cases in the main follow-up. The associations of all predicted carotenoid scores (except for lycopene) with advanced AMD were slightly attenuated (Supplementary Figure 1.3). Neither stopping updating the diet after the date of initial diagnosis of AMD nor further using z-score to account for possible temporal trends of carotenoid intakes materially deattenuated the associations. On the other hand, the associations of all predicted carotenoid scores with intermediate AMD were still null across 4 analytical methods (Supplementary Figure 1.3).

Because statistical methods were unable to fully control for the reverse causation bias possibly due to the fact that AMD patients may have varying degree of diet change depending on initial disease severity, we decided not to include recontacted AMD cases in the main analysis.

IV. Discussion

Our findings from two large, long-running prospective cohorts with repeated dietary assessments suggest that a higher intake of bioavailable lutein/zeaxanthin is associated with a 40% lower risk of advanced AMD. Higher intakes of other bioavailable carotenoids also contribute to a reduced risk of advanced AMD. In contrast, intakes of carotenoids were not associated with intermediate AMD, suggesting an effect on AMD progression rather than initiation.

Although the inverse association between lutein/zeaxanthin and advanced AMD was consistent with a number of previous studies,[12,13,19-21,35] the observational nature of our study precludes the level of causal inference that could be derived from a randomized trial. Unfortunately, the primary analyses of the AREDS 2 trial failed to prove a protective effect of lutein/zeaxanthin.[16] However, when restricted to participants at the bottom 20% of dietary intake of lutein/zeaxanthin, there was a 26% risk reduction.[17] The subgroup result is consistent with the hypothesis that supplements may be more effective when the background dietary intake is below a biologically sufficient threshold. Given the unlikely occurrence of another well-designed large-scale randomized trial, long-running large prospective cohort studies like ours provide the best available evidence to further strengthen the evidence base for a protective role of lutein/zeaxanthin.

Lutein and zeaxanthin form macular pigments that may protect against AMD by reducing oxidative stress, absorbing blue light and stabilizing cell membranes.[11] Cross- sectional

(reviewed in Beatty et al[36]) and experimental studies[37-39] have shown a significant correlation between serum lutein and zeaxanthin and macular pigment optical density (MPOD). Increasing evidence also suggests that genetic variants related to lutein and zeaxanthin metabolism are associated with MPOD or AMD.[40-43] Therefore, multiple independent lines of evidence all point to a protective role of lutein and zeaxanthin in the development of advanced AMD.

Several mechanisms could explain the protective roles of other carotenoids including α-carotene, β-carotene and β-cryptoxanthin, those non-macular pigment carotenoids. All carotenoids are potent antioxidants, which could reduce systematic oxidative stress that indirectly influences the macula. The original AREDS formula containing β-carotene, antioxidant vitamins and minerals but not lutein and zeaxanthin reduced the risk of AMD progression by a quarter.[44] Carotenoids including α-carotene, β-carotene and lycopene have been found in human RPE/choroid,[18] and could protect this tissue against light-induced oxidative damage and locally produced free radicals. The integrity of RPE/choroid could further impact the uptake of lutein/zeaxanthin by retina from the circulating blood. We also speculate that other carotenoids may directly protect lutein/zeaxanthin from oxidative damage in both blood and RPE/choroid. Among a subsample of women in the NHS, we found that measured plasma lutein/zeaxanthin could be significantly predicted by every other plasma carotenoid apart from its own food sources (all $p<0.001$), in accordance with a separate study.[45]

We did not find an association between carotenoids and intermediate AMD. While one previous case-control study[13] and one cross-sectional study[46] reported an inverse association, only one[35] of three prospective cohort studies[15,35,47] reported a significant inverse association between intake of lutein/zeaxanthin and intermediate AMD. One nested case-control study based on 41 cases found

an inverse association for serum lutein/zeaxanthin but not for other carotenoids,[48] whereas two other case-control studies[14,20] only found an inverse association for serum lycopene.

Our study has some limitations. Although our results did not appreciably change after adjusting for many known and suspected risk factors including aHEI, an indicator of a healthy dietary pattern,[49] residual confounding from unaccounted or imprecise measurement cannot be excluded. However, similar associations among ever and never smokers assured us that results were unlikely to be confounded by smoking, the strongest modifiable risk factor for advanced AMD.[50] Because our nutrient and blood database assessed lutein and zeaxanthin together, we were unable to estimate the individual effect of each nutrient to inform the optimal ratio for supplementation. Although the relationship between lutein/zeaxanthin and advanced AMD was linear within the range consumed in our cohorts (0.8 - 10.7 mg/d), we could not evaluate the effect of the higher dosage (10 mg lutein plus 2 mg zeaxanthin) used in the AREDS2 formula. Some patients with intermediate AMD in the later follow-up may have been taking the AREDS formula, which was not ascertained by our FFQs and this may have resulted in underestimation of the true associations between carotenoids and advanced AMD because dietary effects of carotenoids could be masked under intake of pharmacological doses of antioxidant vitamins and minerals.

Strengths of this study included a prospective cohort design with high follow-up that minimized recall and selection biases. Another strength lies in our creation of predicted plasma carotenoid scores to better estimate the true variation of carotenoid exposures accounting for variations in bioavailability across different foods,[51,52] preparation methods,[53] accuracy of responses to various FFQ items, and of food composition databases. Analyses using the predicted plasma scores strengthened the association between lutein/zeaxanthin and advanced AMD, and modestly improved associations with other carotenoids. Differences in the impact of substituting estimated

bioavailable nutrient levels among specific carotenoids might be attributable to variation in the accuracy of FFQ data for specific food items. The predicted plasma score for lutein/zeaxanthin (median, 16.9 μg/ dL) in our cohorts was comparable to the baseline serum level in AREDS2 participants (mean, 17.9 μg/ dL) and the general population participants older than 60 years sampled from 2005-2006 NHANES (mean, 15.0 μg/ dL).[16]

In conclusion, higher intakes of bioavailable carotenoids, particularly lutein/zeaxanthin and α-carotene are associated with reduced risk of advanced AMD. This study lends further support to the causal role of lutein/zeaxanthin in protecting against the development of advanced AMD. Because other carotenoids may also have a protective role, a public health strategy of increasing the consumption of a wide variety of fruits and vegetables rich in carotenoids could be most beneficial and is compatible with current dietary guidelines.

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VI. Tables and Figures

Table 1.1. Age-standardized characteristics of participants in the NHS and HPFS according to the cumulative average predicted plasma score of lutein/zeaxanthin in 1996

	lutein/zeaxanthin, quintiles				
Characteristics	Q1	Q2	Q3	Q4	Q5
NHS					
Participants, No.	10,548	10,548	10,548	10,548	10,548
Age, mean, y	62	62	62	62	63
BMI[¶], mean, kg/m^2	27	27	27	27	26
White race, %	99	98	98	98	97
Current smokers, %	17	13	10	9	8
Pack years of smoking	29	25	23	21	20
Physical activity, median, MET-h/wk	8	9	11	13	16
Hypertension, %	39	41	41	41	38
Postmenopausal, %	90	90	90	90	90
Current menopausal hormone use[§], %	37	41	42	43	44
Current aspirin use, %	46	48	50	50	50
Dietary intake, mean					
Total energy intake, Kcal/d	1,703	1,670	1,698	1,756	1,877
Alcohol, g/d	5	5	6	6	7
Fruits and vegetables, servings/d	4	5	5	6	8
aHEI (excluding fruits and vegetables)	39	41	42	43	44
Lutein/zeaxanthin, µg/d	1,657	2,259	2,732	3,338	4,779
α-carotene, µg/d	528	662	767	873	1,112
β-carotene, µg/d	2,890	4,690	4,324	5,101	6,862
β-cryptoxanthin, µg/d	110	156	184	208	241
Lycopene, µg/d	5,935	6,314	6,571	6,748	7,093
ALA, g/d	0.93	0.95	0.96	0.98	1.01
DHA, g/d	0.11	0.13	0.14	0.16	0.19
Predicted lutein/zeaxanthin plasma score, mean, µg/L	152	161	169	179	203
HPFS					
Participants, No.	4,999	4,999	5,000	4,999	4,999
Age, mean, y	62	63	63	64	64
BMI[¶], mean, kg/m^2	26	26	26	26	26
White race, %	97	96	95	96	95
Current smokers, %	8	5	4	4	4
Pack years of smoking	14	13	12	12	11
Physical activity, median, MET-h/wk	20	23	25	28	32
Hypertension, %	34	34	34	33	34
Current aspirin use, %	68	70	70	68	67
Dietary intake, mean					
Total energy intake, Kcal/d	2,068	1,938	1,956	1,998	2,115

Table 1.1. (Continued) Age-standardized characteristics of participants in the NHS and HPFS according to the cumulative average predicted plasma score of lutein/zeaxanthin in 1996

	lutein/zeaxanthin, quintiles				
Characteristics	Q1	Q2	Q3	Q4	Q5
Alcohol, g/d	12	11	11	11	11
Fruits and vegetables, servings/d	4	5	6	6	8
aHEI (excluding fruits and vegetables)	41	43	44	45	47
Lutein/zeaxanthin, μg/d	1,848	2,563	3,091	3,832	5,468
α-carotene, μg/d	606	768	879	1,024	1,374
β-carotene, μg/d	3,413	4,341	5,052	6,013	8,152
β-cryptoxanthin, μg/d	121	172	211	244	307
Lycopene, μg/d	6,545	7,223	7,532	7,888	8,520
ALA, g/d	1.05	1.07	1.08	1.10	1.13
DHA, g/d	0.16	0.19	0.20	0.23	0.27
Predicted lutein/zeaxanthin plasma score, mean, μg/L	149	159	167	177	202

Abbreviations: BMI, body mass index; MET-h, hours of metabolic equivalent tasks; aHEI, alternative healthy eating index; ALA, α-linolenic acid; DHA, docosahexaenoic acid.

[¶]BMI is calculated as weight in kilograms divided by height in meters squared.

[§]Current menopausal hormone use among postmenopausal women.

Table 1.2. Pooled hazard ratios of AMD according to quintiles of predicted plasma carotenoid scores and calculated intakes in NHS and HPFS

	Advanced AMD		Intermediate AMD	
	Predicted Plasma Score	Calculated Intake[¶]	Predicted Plasma Score	Calculated Intake[¶]
Carotenoids	Multivariate HR (95% CI)			
Lutein/Zeaxanthin				
Q1	1(Ref.)	1(Ref.)	1(Ref.)	1(Ref.)
Q2	0.82 (0.68, 0.99)	0.90 (0.75, 1.08)	0.97 (0.82, 1.15)	0.92 (0.78, 1.10)
Q3	0.94 (0.78, 1.12)	0.86 (0.71, 1.03)	0.95 (0.80, 1.13)	0.96 (0.81, 1.14)
Q4	0.83 (0.69, 1.00)	0.81 (0.67, 0.99)	0.93 (0.78, 1.11)	0.96 (0.80, 1.14)
Q5	0.59 (0.48, 0.73)	0.79 (0.64, 0.97)	0.93 (0.78, 1.12)	0.97 (0.81, 1.16)
p for trend	<.001	0.04	0.42	0.99
p for heterogeneity	0.99	0.04	0.92	0.17
β-cryptoxanthin				
Q1	1(Ref.)	1(Ref.)	1(Ref.)	1(Ref.)
Q2	0.94 (0.78, 1.14)	0.83 (0.69, 1.01)	0.87 (0.73, 1.04)	0.97 (0.81, 1.15)
Q3	0.87 (0.72, 1.05)	0.86 (0.72, 1.04)	1.00 (0.84, 1.18)	0.98 (0.82, 1.16)
Q4	0.90 (0.74, 1.08)	0.84 (0.70, 1.02)	0.91 (0.77, 1.09)	0.90 (0.75, 1.08)
Q5	0.73 (0.60, 0.89)	0.71 (0.58, 0.86)	0.85 (0.72, 1.02)	0.90 (0.75, 1.07)
p for trend	0.002	0.002	0.12	0.12
p for heterogeneity	0.97	0.73	0.20	0.13
Lycopene				
Q1	1(Ref.)	1(Ref.)	1(Ref.)	1(Ref.)
Q2	0.98 (0.83, 1.16)	1.02 (0.86, 1.21)	0.94 (0.81, 1.10)	0.98 (0.84, 1.15)
Q3	0.93 (0.78, 1.11)	0.97 (0.81, 1.16)	1.01 (0.86, 1.19)	0.98 (0.83, 1.15)
Q4	0.85 (0.71, 1.03)	0.77 (0.63, 0.94)	0.95 (0.80, 1.12)	1.04 (0.88, 1.23)
Q5	0.93 (0.76, 1.13)	1.00 (0.83, 1.21)	1.04 (0.87, 1.23)	1.05 (0.88, 1.24)
p for trend	0.17	0.38	0.64	0.44
p for heterogeneity	0.08	0.59	0.86	0.96
α-carotene				
Q1	1(Ref.)	1(Ref.)	1(Ref.)	1(Ref.)
Q2	0.99 (0.82, 1.19)	0.81 (0.67, 0.98)	0.88 (0.73, 1.04)	0.94 (0.78, 1.13)
Q3	0.92 (0.76, 1.11)	0.82 (0.68, 0.99)	0.91 (0.77, 1.08)	0.95 (0.79, 1.13)
Q4	0.88 (0.73, 1.07)	0.82 (0.68, 0.99)	0.87 (0.73, 1.04)	1.13 (0.95, 1.34)
Q5	0.69 (0.56, 0.84)	0.68 (0.56, 0.83)	0.94 (0.79, 1.12)	0.98 (0.82, 1.17)
p for trend	<.001	0.002	0.86	0.69
p for heterogeneity	0.36	0.08	0.68	0.24
β-carotene				
Q1	1(Ref.)	1(Ref.)	1(Ref.)	1(Ref.)
Q2	0.97 (0.80, 1.17)	1.04 (0.86, 1.26)	1.02 (0.85, 1.22)	0.99 (0.82, 1.18)
Q3	0.90 (0.74, 1.09)	0.96 (0.79, 1.16)	1.08 (0.90, 1.29)	1.09 (0.91, 1.30)
Q4	0.80 (0.65, 0.97)	0.86 (0.70, 1.05)	0.99 (0.82, 1.19)	1.02 (0.85, 1.23)
Q5	0.82 (0.67, 1.01)	0.86 (0.69, 1.06)	1.03 (0.85, 1.24)	0.99 (0.82, 1.20)

Table 1.2. (Continued) Pooled hazard ratios of AMD according to quintiles of predicted plasma carotenoid scores and calculated intakes in NHS and HPFS

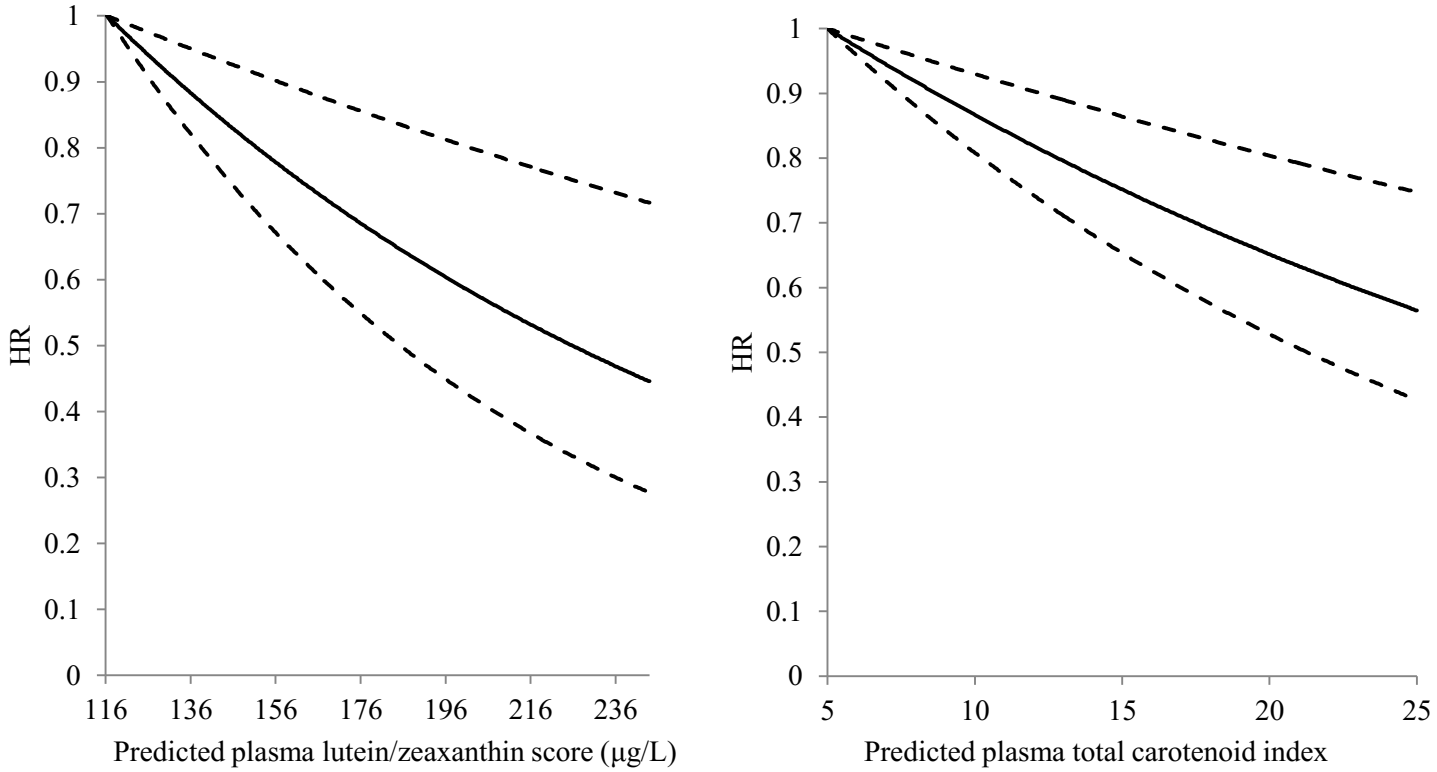
	Advanced AMD		Intermediate AMD	
	Predicted Plasma Score	Calculated Intake¶	Predicted Plasma Score	Calculated Intake¶
Carotenoids	Multivariate HR (95% CI)			
p for trend	0.03	0.05	0.92	0.88
p for heterogeneity	0.28	0.99	0.87	0.65
β-carotene from food				
Q1	1(Ref.)	1(Ref.)	1(Ref.)	1(Ref.)
Q2	0.84 (0.69, 1.02)	0.97 (0.80, 1.18)	0.93 (0.77, 1.11)	0.86 (0.71, 1.03)
Q3	0.80 (0.66, 0.97)	0.89 (0.73, 1.09)	0.97 (0.80, 1.16)	0.96 (0.80, 1.15)
Q4	0.79 (0.64, 0.96)	0.86 (0.70, 1.05)	1.00 (0.83, 1.21)	1.01 (0.84, 1.21)
Q5	0.64 (0.52, 0.79)	0.68 (0.55, 0.85)	1.02 (0.84, 1.24)	0.92 (0.76, 1.12)
p for trend	<.001	<.001	0.47	0.88
p for heterogeneity	0.67	0.60	0.94	0.90
Total carotene from food				
Q1	1(Ref.)	1(Ref.)	1(Ref.)	1(Ref.)
Q2	0.89 (0.74, 1.08)	0.97 (0.80, 1.17)	0.88 (0.73, 1.06)	0.87 (0.72, 1.05)
Q3	0.76 (0.62, 0.93)	0.89 (0.74, 1.09)	0.95 (0.79, 1.14)	0.98 (0.82, 1.17)
Q4	0.77 (0.63, 0.94)	0.78 (0.64, 0.95)	0.94 (0.78, 1.14)	0.96 (0.80, 1.16)
Q5	0.64 (0.51, 0.79)	0.72 (0.58, 0.89)	0.99 (0.82, 1.19)	0.94 (0.78, 1.13)
p for trend	<.001	<.001	0.64	0.89
p for heterogeneity	0.59	0.65	0.96	0.40
Total carotenoid index§				
Q1	1(Ref.)	1(Ref.)	1(Ref.)	1(Ref.)
Q2	0.95 (0.80, 1.14)	0.83 (0.69, 1.00)	0.77 (0.64, 0.91)	0.95 (0.80, 1.13)
Q3	0.84 (0.70, 1.01)	0.82 (0.68, 0.99)	0.93 (0.79, 1.10)	0.97 (0.82, 1.15)
Q4	0.77 (0.64, 0.94)	0.79 (0.65, 0.95)	0.87 (0.73, 1.03)	1.06 (0.90, 1.26)
Q5	0.65 (0.53, 0.80)	0.72 (0.59, 0.87)	0.92 (0.77, 1.10)	0.99 (0.83, 1.18)
p for trend	<.001	0.001	0.80	0.73
p for heterogeneity	0.67	0.51	0.98	0.56

Multivariate models were adjusted for: age (continuous), BMI (<18.5, 18.5-23, 23-25, 25-30, 30-35, ≥ 35 kg/m2), pack-years of smoking (never, 1-9, 10-24, 25-44, 45-64, ≥65y), physical activity (<3, 3-8.9, 9-17.9, 18-26.9, ≥27 MET-h/wk), current aspirin use (≥1 tablets/wk or none), history of hypertension, diabetes and cardiovascular diseases, dietary variables including aHEI (excluding fruits and vegetables), alcohol intake, DHA and ALA (all in quintiles). In NHS, models were additionally adjusted for postmenopausal status and menopausal hormone use (never, current and past); in HPFS additional adjustment for race (Caucasian v.s. non-Caucasian).

§Total carotenoid index was created by summing the quintile score of each carotenoid.

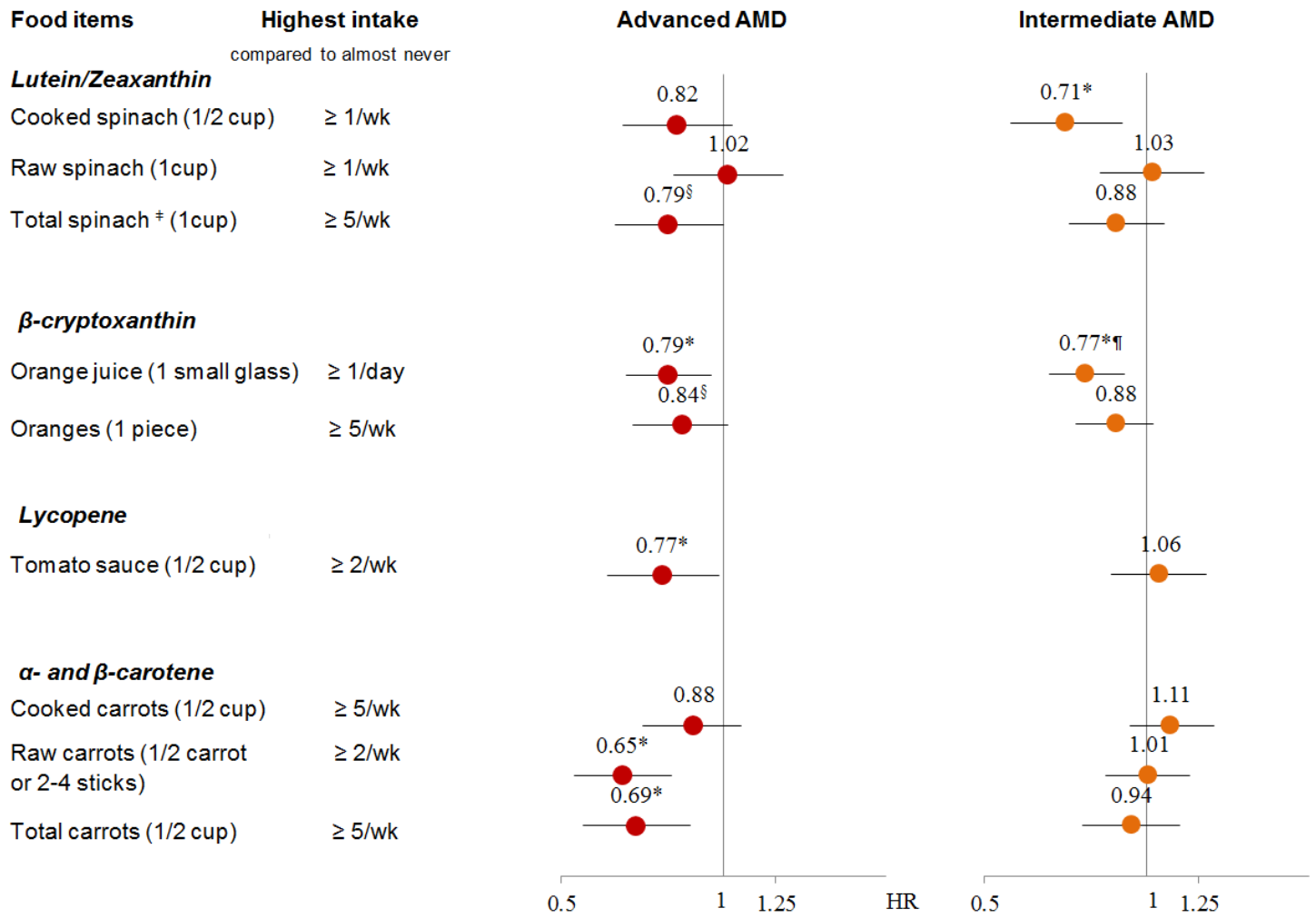
¶ Multivariate models were additionally adjusted for total calorie intake (in quintiles).

Figure 1.1. Dose-response relationship between predicted plasma carotenoid scores and the risk of advanced AMD



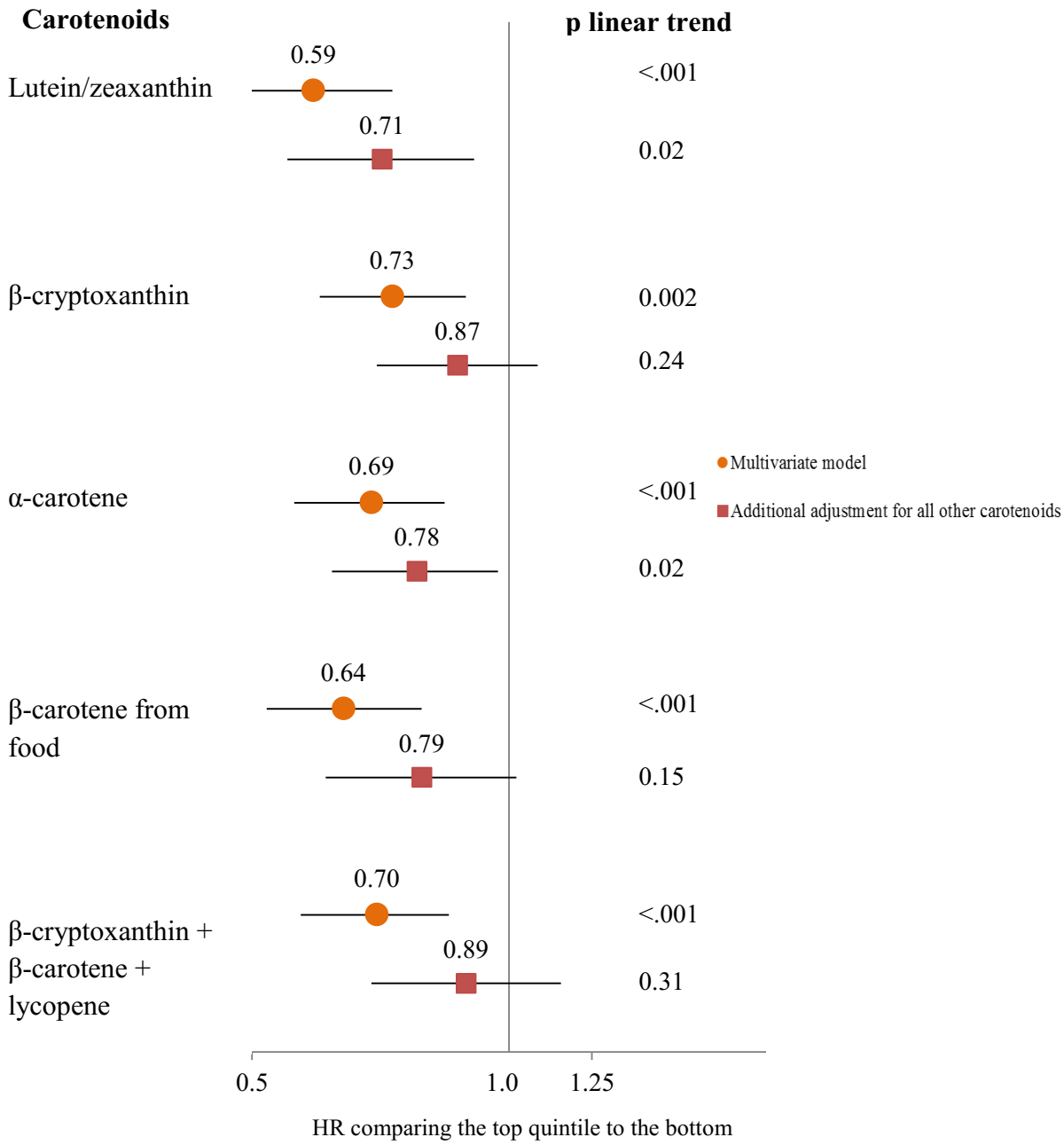
Multivariate models were adjusted for: age (continuous), BMI (≥30 kg/m^2), current aspirin use (≥1 tablets/wk), history of hypertension, pack-years of smoking, physical activity, and modified aHEI (all in categories).
The solid lines represent the HRs and the dotted lines represent the 95% CIs.

Figure 1.2. Hazard ratios of AMD according to primary carotenoid-containing foods



*, p for linear trend < 0.05; §, p for linear trend <0.10
¶, p for heterogeneity between NHS and HPFS <0.05
‡, Cooked spinach (1/2 cup) ≅ 2.5 * raw spinach (1 cup); Total spinach (1 cup) = raw spinach (1 cup) + 2.5* cooked spinach (1/2 cup).
Multivariate models were adjusted for the same variables as in the Table 1.2.

Figure 1.3. Independent associations of predicted plasma carotenoid scores with advanced AMD



All other carotenoids were a composite score derived by totaling the quintile score of each carotenoid.
p for heterogeneity between NHS and HFPS was > 0.10 for all the HRs.
Multivariate models were adjusted for the same variables as in the Table 1.2.

CHPATER II

DIETARY INTAKES OF EICOSAPENTAENOIC ACID AND DOCOSAHEXAENOIC ACID AND RISK OF AGE-RELATED MACULAR DEGENERATION

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ABSTRACT

Objectives: To evaluate the associations between intake of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the intermediate and advanced stages of AMD.

Design: Prospective cohort study.

Participants: 75,889 women from the Nurses' Health Study and 38,961 men from the Health Professionals Follow-Up Study were followed from 1984 to 2012 and 1986 to 2010, respectively. At baseline all participants were free of diagnosed AMD and major chronic diseases (diabetes, cardiovascular disease and cancer) that may lead to a change of habitual diet.

Methods: We assessed dietary intake by a validated food frequency questionnaire (FFQ) at baseline and every four years. We calculated cumulative average intake of EPA and DHA from FFQs and also computed predicted erythrocyte and plasma scores directly from food intake using regression models. Cox proportional hazards models were used to compute the associations with AMD outcomes.

Main Outcome Measures: We confirmed 1,589 incident intermediate and 1,356 advanced AMD cases (primarily neovascular AMD) by medical record review.

Results: For intermediate AMD, the pooled hazard ratio between the two cohorts for DHA comparing the extreme quintiles of intake was 0.78 (95% CI = 0.66 - 0.92; p trend = 0.008) and for EPA + DHA was 0.83 (95% CI = 0.71 - 0.98; p trend = 0.03). The pooled hazard ratio for fatty fish comparing ≥ 5 servings/wk to almost never was 0.61 (95% CI = 0.46 - 0.81; p trend < 0.001). Secondary analyses using predicted erythrocyte and plasma scores of EPA and DHA

yielded similar findings. For advanced AMD, we found no associations in the pooled analysis for either intake or predicted bioavailable levels.

Conclusion: Higher intakes of EPA and DHA may prevent or delay the occurrence of visually significant intermediate AMD. However, the totality of current evidence for EPA and DHA and advanced AMD is discordant, though there was no association with advanced AMD in the present study.

I. Introduction

Age-related macular degeneration (AMD) is a chronic, degenerative disease of the macula, which can result in loss of the ability to read, write or drive.[1] Although the advanced stages of AMD are often debilitating, some forms of advanced AMD can now be successfully managed with intravitreal injection of anti-VEGF agents, allowing patients to maintain or even restore vision for variable periods of time.[2-4] On the other hand, although usually less visually debilitating, early/intermediate AMD affects a much larger number of persons worldwide, and increases risk of development of advanced AMD. According to the 2005-2008 US National Health and Nutrition Examination Survey (NHANES), the estimated prevalence in persons aged 40 years and older was 5.7% for early/intermediate AMD versus 0.8% for advanced AMD.[5] Globally, among people of European ancestry, the prevalence was 11.2% for early/intermediate AMD versus 0.5% for advanced AMD.[6] Although decreasing exposure to some risk factors (e.g. smoking and blood pressure) in recent years might eventually help mitigate the incidence of early/intermediate AMD,[5] owing to rapidly aging populations and lack of other effective means of primary prevention, the number of early/intermediate AMD cases is expected to double in the next few decades.[6-8] Therefore, identification of other means of primary prevention, especially for vision-threatening early/intermediate AMD cases, would carry marked public health significance.

Docosahexaenoic acid (DHA), a long-chain omega3 (n3) fatty acid, is a major lipid component of retinal photoreceptor outer segment membranes that has anti-inflammation and anti-angiogenesis properties that could protect against AMD.[9,10] The retinal concentration of DHA is dependent upon and modifiable by diet.[11] Eicosapentaenoic acid (EPA), although not concentrated in the retina, is a precursor to DHA, and its metabolites could similarly affect the

pathogenic processes of AMD.[9] Our earlier study[12] and investigations by others[13-15] suggested that long-chain n3 fatty acids (DHA, EPA, and other 20 and 22-carbon n3 fatty acids) may reduce the risk of early/intermediate AMD. With respect to advanced AMD, intake of DHA was inversely associated with advanced AMD in several prospective cohort studies;[13,16-18] but this finding was not corroborated by the Age-Related Eye Disease Study 2 (AREDS2) trial in which supplementation with DHA and EPA for 5 years did not slow the progression to advanced AMD among patients with intermediate AMD.[19]

In light of the mixed findings from prior literature, we aimed to evaluate the relations of intakes of EPA and DHA to different stages of AMD in large prospective cohorts over 28 years of follow-up.

II. Methods

Study Population

The two large ongoing US prospective cohorts, the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS), have been described in detail before.[20,21] Briefly, the NHS includes 121,701 US female registered nurses aged 30 to 55 years in 1976. The HPFS includes 51,529 US male health professionals aged 40 to 75 years in 1986. Both cohorts are predominantly white (NHS: >98%; HPFS: >91%). The long-term follow-up rates are >95%. Questionnaires were mailed to all participants biennially to inquire updated lifestyle factors and disease outcomes. Food frequency questionnaires (FFQ's) were mailed every four years to assess diet in the preceding year. Submission of a completed self-administered questionnaire was deemed to imply informed consent. The study protocol was approved by the Institutional Review Boards at the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health.

At study baseline (1984 in the NHS and 1986 in the HPFS), we excluded participants who did not return the initial FFQ, left more than 70 food items blank, reported implausible dietary intake (<600 or >3500 Kcal/d for the NHS and <800 or > 4200 Kcal/d for the HPFS), had prevalent AMD, or serious chronic diseases including cancer (except nonmelanoma skin cancer), diabetes and cardiovascular disease. To minimize detection bias, we also excluded participants who never reported an eye exam over the entire follow-up and excluded from analysis the person-time during any two-year interval in which a participant did not report an eye exam. Results did not materially change in sensitivity analyses including intervals lacking an eye exam. Participants were included in the analysis when $\geqslant$ 50 years old, and were censored at age 90 to alleviate concerns of low reporting validity (NHS, n=15; HPFS, n=528). By the end of follow-up, a total of 75,889 women and 38,961 men contributed to the analysis.

AMD Ascertainment

Our case definition has been previously validated by comparison with retinal images and medical records.[22] When a participant reported a diagnosis of AMD on a biennial questionnaire, we requested informed consent and then contacted the participant's eye doctor to confirm the diagnosis by review of medical records. Cases with only small hard drusen (< 63μm diameter) were excluded. We defined intermediate AMD as having at least one of the following signs: intermediate drusen (≥ 63 and <125 μm), pigment abnormalities, large drusen (≥ 125 μm) or any non-central geographic atrophy (GA). We defined neovascular AMD as having any of the following: RPE detachment, subretinal neovascular membrane, disciform scar, or history of treatment with laser, photodynamic, or anti-VEGF therapies for AMD. Central GA was defined as having a central geographic atrophy lesion involving the center of the macula. Advanced AMD included neovascular AMD and central GA. Additionally, all case definitions, except

those recent neovascular AMD cases that had anti-VEGF therapies, included a visual acuity of 20/30 or worse due primarily to AMD. This magnitude of vision loss is not only of clinical significance, but also is severe enough to warrant medical attention so as to minimize potential detection bias arising from differential health consciousness. The person was used as the unit of analysis, and the worst eye was used for classification.

Dietary Assessment

We began follow up in 1984 for the NHS and 1986 for the HPFS, when the first comprehensive FFQ with an expanded section on fish was administered, and assessed dietary intake every four years thereafter. FFQ items on fish or seafood consumption includes 1) canned tuna (3-4 oz); 2) dark meat fish (3-5 oz); 3) other fish (mainly white fish, 3-5 oz); 4) shrimp, lobster or scallops as main dish (3-5 oz). On the FFQs, commonly used units or portion sizes (e.g., 1 orange or ½ cup broccoli) are specified for the approximately 130 items. Participants were asked to report how often, on average over the past year, they had consumed each food item (responses ranging from "≤1 time per month" to "≥6 times/day"). Fish oil supplements including marine and cod liver oils were assessed from 1990 in the NHS and 1988 in the HPFS. We calculated nutrient intakes by multiplying the consumption frequency of each food by the nutrient content of the specified food portion summing across all foods. The nutrient composition data were primarily based on the US Department of Agriculture Nutrient Database supplemented with information from manufacturers and published reports. Nutrient values were energy-adjusted using the residual method.[23]

The validity and reproducibility of FFQs in measuring polyunsaturated fatty acids and fish intake has been assessed in a random sample of 118 HPFS participants who completed two consecutive

FFQs (1986 & 1987), two 1-week dietary records ~ 7 months apart and provided subcutaneous fat samples.[24] The correlation was 0.61 for fish between two FFQs.[25] The correlation between energy-adjusted EPA from FFQs and percentage of EPA in the adipose tissue was 0.47.[24] An earlier validation study in the NHS cohort had similar findings.[26,27]

Measurement of Erythrocyte and Plasma EPA and DHA

Measurement error in FFQs and imprecision in the nutrient composition database may introduce error into the calculated intake of EPA and DHA. Because erythrocyte and plasma EPA and DHA reflect long-term dietary intake,[28] we used an empirical prediction model to predict the erythrocyte and plasma levels of EPA and DHA directly from food intake based on previous blood measurements among participants of nested case-control studies of cardiovascular disease in the NHS and HPFS. We included all the cases and controls because all were free of disease at the time of blood collection. The details on blood collection and measurements have been described previously.[28,29] Briefly, we collected whole blood samples from 32,826 women between 1989 and 1990 and from 18,225 men between 1993 and 1995. Risk set sampling was used to select 1-2 controls for each confirmed cases matched on age (within 2 years), time of blood donation, and other factors (e.g. smoking and fasting status) from 1990 to 2006 in the NHS and 1994 to 2004 in the HPFS.

Statistical Analysis

Participants contributed person-time to the analysis from the return of the baseline questionnaire if over age 50 years at baseline or from reaching 50 years old to the confirmed diagnosis of AMD, death, loss to follow-up or the end of follow-up (05/31/2012 for the NHS and 01/31/2010 for the HPFS), whichever occurred first. To best represent long-term intake and minimize

measurement error,[30] we calculated the cumulative average of intakes of EPA and DHA. All cumulative averages were categorized into quintiles based on the distribution in each cohort.

We used time-varying Cox proportional hazards model to estimate the hazard ratio (HR) and 95% confidence interval (CI) controlling for known and suspected risk factors, including race, body mass index (BMI), pack-years of smoking, physical activity, aspirin use, history of hypertension, history of hypercholesterolemia, menopausal status and postmenopausal hormone use (in the NHS only), and the alternative Healthy Eating Index [31] (aHEI, modified by excluding EPA + DHA) to account for confounding by healthy dietary pattern. We further adjusted for α-linoleic acid (ALA), the 18-carbon n3 fatty acid that was positively associated with AMD in our cohorts.[12] We also tested whether the associations would vary by intake of linoleic acid (LA), an omega6 fatty acid, and by age. We created binary variables using the median intake of LA in each cohort, and the median age of onset of AMD cases (73 years old). We created the interaction terms and used a likelihood ratio test to test the significance of interactions by comparing models with and without the interaction terms.

The empirical prediction model has been described previously.[32] Briefly, among food sources of EPA and DHA, we used stepwise linear regression to select foods that were significantly predictive of EPA and DHA blood measurements ($p < 0.05$). We used the average of food intake between 1986 and 1990 FFQs in the NHS and between 1990 and 1994 FFQs in the HPFS to correspond with the time of blood draw and to reduce within-person variation. The prediction models were separately created in each cohort. We specifically excluded fish oil supplement users so that the predicted blood scores, when extended to all cohort participants, would more accurately reflect long-term dietary intake because intake of fish oil supplements during the follow-up was intermittent (only 4% participants in each cohort had a consistent intake for ≥ 4

years). We then computed predicted scores based on food intake from the FFQ at each 4-year cycle for the full cohorts and used the cumulative average values in the analysis.

We performed the analyses separately in each cohort using SAS 9.3. To derive a pooled HR, we first combined the two cohorts and then used a Cox proportional hazards model in the pooled data stratified by the cohort. Interpretation of the results was mainly based on pooled HRs unless otherwise specified. All hypothesis tests were two-sided and used an α-value of 0.05.

III. Results

In 1998 (the middle of follow-up), participants at the highest cumulative average intake of EPA + DHA were likely to be more physically active, smoke less and consume a healthier diet. However, they were more likely to have a history of hypertension and hypercholesterolemia (Table 2.1).

Intakes of EPA and DHA were strongly correlated (Pearson r=0.88). During the follow-up, intake of food-sourced EPA + DHA declined slightly (and the same for fatty fish), whereas the intake of fish oil supplements increased markedly especially after 2002 (Figure 2.1).

1,589 intermediate AMD cases (1,209 in the NHS and 380 in the HPFS) and 1,356 advanced cases (1,010 in the NHS and 346 in the HPFS) were included in the analysis, and 96% of the advanced AMD cases were neovascular types. In pooled primary analysis (Table 2.2), the HR comparing extreme quintiles of intake of EPA + DHA for risk of intermediate AMD was 0.83 (95% CI = 0.71 – 0.89; p trend = 0.03) but the inverse association was primarily attributable to DHA (HR=0.78; 95% CI = 0.66 – 0.92; p trend=0.008). Results excluding fish oil supplement users were similar. With respect to advanced AMD, we did not find any associations in the pooled analysis, with the exception of a significant inverse association in the HPFS for intake of

EPA + DHA when excluding fish oil supplement users. The results for advanced AMD were not materially altered in sensitivity analyses excluding African American participants, as they generally have a lower risk of AMD,[1,33,34] as well as in analyses using deciles of intake (data not shown).

We further examined associations between more remote dietary intake with AMD using the average of the first two FFQs in case an association with AMD would be missed if there was a long latency period between the intake and AMD onset. However, remote intakes of EPA and DHA were not associated with either intermediate or advanced AMD (data not shown).

Supplementary Table 2.1 provides the empirical prediction models used for computing predicted erythrocyte and plasma scores of EPA and DHA for all cohort participants. The Spearman correlations between predicted blood scores and measured blood levels ranged from 0.21 to 0.56 with stronger correlations for DHA and for measurements in the HPFS (Supplementary Table 2.2). Among non-users of fish oil supplements, the pooled HRs of intermediate AMD for DHA were similar across analyses based on diet, predicted erythrocyte and plasma scores; the associations for EPA were strengthened when using predicted erythrocyte and plasma scores (Figure 2.2). With respect to advanced AMD, although the pooled HRs were similar across analyses, we observed significant heterogeneity between the HRs in the NHS and HPFS. In the HPFS, there were significant inverse associations for predicted erythrocyte and plasma scores of EPA and DHA with advanced AMD (Supplementary Table 2.3).

Intake of total fatty fish was significantly inversely related to intermediate AMD (p for trend across intake categories < 0.001); the association was primarily attributed to canned tuna (Table 2.3), for which the median intake was 3.5 times that of dark oily fish. In contrast, intake of other

fish (mainly white fish) was not associated with the risk of intermediate AMD. We did not find any significant association between any type of fish and advanced AMD.

Two percent of the population in each cohort had a consistent intake of fish oil supplements for ≥ 6 years. In terms of intermediate AMD, in the NHS after controlling for food-sourced intake of EPA + DHA and other risk factors, those consistent users compared to irregular users and non-users had a 40% (HR = 0.60; 95% CI = 0.36 – 1.01; p = 0.05) lower risk. When further restricting the comparison to consistent versus irregular users, the risk reduction was 45% (HR = 0.55; 95% CI = 0.32 – 0.93; p = 0.02). With respect to advanced AMD, in the NHS consistent users compared to irregular users did not have a reduced risk (HR = 1.07; 95% CI = 0.73 – 1.58). In contrast, there were no significant associations in the HPFS between fish oil supplements and any type of AMD, but the 95% CIs surrounding the HRs were wide (e.g. for intermediate AMD comparing consistent to irregular users, HR = 0.95; 95% CI = 0.45 – 1.98; p = 0.88; other data not shown).

High intake of n6 fatty acids is hypothesized to negate the inverse associations for long-chain n3 fatty acids due to competition for the same enzymes.[16,35,36] To explore this possibility, we stratified the analyses by the median intake level of linoleic acid, a major omega6 fatty acid. We did not find any statistically significant interactions in the pooled analysis (Supplementary Table 2.4). We also did not find any significant interactions by age, although the HRs between DHA and intermediate AMD seemed to be lower in younger people in the NHS but had an opposite pattern in the HPFS (Supplementary Table 2.5).

IV. Discussion

In this large prospective study with 24-28 years of follow-up, high intake of DHA + EPA (especially DHA) and fatty fish, was associated with a 17 to 40% lower risk of visually significant intermediate AMD, but no reduction in the risk of advanced AMD. Results based on predicted erythrocyte and plasma DHA and EPA scores supported observations based on dietary intake. Overall, these data support the hypothesis that DHA and EPA may prevent or delay the onset of AMD.

Among existing studies on the relation between intake of long-chain n3 fatty acids or fish with early/intermediate AMD, , two[37,38] out of four cross-sectional studies[37-40] reported an inverse association whereas 1 case-control study did not.[41] In all 3 prospective cohort studies an inverse association was found.[13-15] In particular, the 10-year prospective Women's Health Study, which has a similar study methodology to ours (e.g. a cohort of female health professionals, the same Willett FFQs and classification of AMD types)[14] showed similar results. Ours is the only study that has further explored and showed an inverse association for a consistent intake of fish oil supplements, although the HR was only significant in the NHS. A much smaller number of cases in the HPFS may have resulted in an inadequate power to detect an association; the wide CI for the HR in the HPFS did include the point estimate in the NHS.

With respect to advanced AMD, an apparent discrepancy exists between observational studies across several populations and randomized trials. Observational studies[13,16-18,35-39] were almost all suggestive of an inverse association, including four prospective cohort studies,[13,16-18] two case-control studies,[35,36] and two [38,39] out of three cross-sectional studies[37-39]. Two cross-sectional studies based on erythrocyte or plasma EPA and DHA among a French population also

suggested an inverse association.[42,43] In contrast, in two double-blinded, placebo-controlled, randomized trials, AREDS2 from US[19] and NAT2 from France,[44] supplementation of high dose EPA and DHA did not reduce the risk of progression to advanced AMD over 3-5 years of follow-up. While confounding may cause the inverse associations in observational studies, several other reasons could explain the null findings in randomized trials, including insufficient dosage, short duration of follow-up, timing of interventions that did not encompass the true latent period, poor compliance (NAT2 trial), a high baseline intakes of EPA and DHA, etc. In this study, although the associations with advanced AMD were null in the pooled analysis, in the HPFS we found significant inverse associations especially when using the predicted biomarker scores. The correlations between calculated intakes of EPA and DHA and measured blood levels were stronger in the HPFS than in the NHS perhaps due to a higher intake of fatty fish in the HPFS. However, the possibility of a chance finding cannot be excluded due to a much smaller number of advanced cases in the HPFS. Therefore, totaling all of existing evidence, the associations between intakes of EPA and DHA with advanced AMD remain unclear.

Intuitively, a reduction in the incidence of intermediate AMD with EPA and DHA could ultimately decrease that of advanced AMD. However, AMD is a complex disease with different clinical manifestations and underlying genetic profiles.[45-47] Clinically-defined intermediate AMD cases could harbor different underlying genetic backgrounds with different propensities for progression. Our AMD ascertainment scheme may have accrued mostly those intermediate AMD cases those that were slow progressing or not destined to progress further. The existence of this subset of intermediate AMD cases is plausible in view of: 1) the high prevalence of intermediate AMD (>20%) at older ages in white populations;[5,7,48] 2) the relatively small proportion of

intermediate AMD that progresses (18% progression rate over 5 years in the AREDS study[49] and 16% over 20 years by our estimation in the Beaver Dam Eye Study[48]).

Multiple biological mechanisms by which EPA and DHA could affect the development of AMD have been proposed.[9] Briefly, EPA and DHA may modulate the immune and inflammatory processes implicated in the pathogenesis of AMD by influencing gene expression, cell differentiation and survival either through affecting cell membrane function or interceding cellular signaling cascades.[9] The metabolites of EPA and DHA, eicosanoids, are also known as potent regulators of immune and inflammatory responses.[50,51]

Our study has several strengths and limitations. The prospective design and high follow-up rate minimized the likelihood of recall and selection bias, respectively. To our knowledge, this is the only observational study that has repeated dietary assessment, which not only dampens within-person variation but also reflects dietary change over time. Also to our knowledge, we are the first study to make use of food intake and existing blood data to develop predicted blood scores for AMD research. The empirical prediction model provides an alternative way of estimating exposure that accounts for the variation in accuracy across self-reported foods, imprecision in food composition database and variation in absorption and metabolism. Consistent findings between blood and dietary analyses lend support to the validity of our results. The observational design cannot exclude residual or unmeasured confounding, but this is unlikely to have a major impact on the current results, as the HRs did not appreciably change after extensive adjustment for known and suspected risk factors. To rule out the possibility that high intake of EPA and DHA is a marker for healthy dietary pattern, we specifically adjusted for a recognized indicator of diet quality,[31] and the results were essentially the same pre and post-adjustment. EPA and DHA could also be a marker for other beneficial components of fatty fish, e.g. vitamin D, but the

suggestive inverse association also seen for fish oil supplements is consistent with the hypothesis that the primary causal factors are EPA and DHA. Another issue of concern is that participants at the high intake of EPA and DHA were more likely to be health-conscious and to have an eye exam, and thus to have existing AMD diagnosed. This could result in detection bias that may particularly affect early/intermediate AMD, which is usually asymptomatic, and may lead to an underestimation of a true benefit. To minimize the effect of such bias, we restricted to those AMD cases that were sufficiently visually significant to warrant medical attention and also excluded those who did not report an eye exam in the past 2 years.

In summary, this prospective study over more than two decades of follow-up supports beneficial effects of EPA and DHA on the risk of visually significant intermediate AMD. Specifically, intake of EPA + DHA ≥ 350 mg/d or fatty fish starting from ≥ 2 servings/week may provide a moderate reduction in risk. Given that less than 5% of US adults and fewer than 1 in 4 adults consumed that amount of EPA + DHA and fatty fish, respectively, according to 2009 – 2010 NHANES,[52] increasing the average intake on a population basis may have implications for the primary prevention of visually significant intermediate AMD. Consistent long-term intake of fish oil supplements, which on average contain 590 mg of EPA + DHA,[53] may be an appealing alternative to achieve the same benefit. Whether intakes of EPA and DHA may protect against the development of advanced AMD is still inconclusive but current evidence does not suggest any harm.

V. References

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VI. Tables and Figures

Table 2.1. Age-standardized characteristics of participants according to cumulative average intake of EPA + DHA in 1998 (middle of follow-up)

	EPA + DHA (diet + supplements), Quintiles				
	Q1	Q2	Q3	Q4	Q5
NHS					
Participants, No.	13,329	13,198	13,268	13,208	13,267
Age, y	63	63	64	64	64
¶BMI, kg/m^2	26.5	26.6	26.8	26.8	26.8
Caucasians, %	98	98	98	97	96
Current smokers, %	12	11	10	9	8
Pack years of smoking	27	25	24	23	22
Physical activity, MET-h/wk	15	16	17	19	21
Hypertension, %	42	43	43	45	46
Hypercholesterolemia, %	54	56	57	59	60
Postmenopausal, %	94	94	94	94	94
§Current menopausal hormone use, %	42	44	46	46	46
Current aspirin use, %	44	48	50	50	50
Fish oil supplements, %	0	0	1	3	12
Dietary intake					
ALA, mg/d	956	962	961	963	978
EPA + DHA, mg/d	67	123	174	240	416
LA, g/d	9	9	9	9	9
Fruits and vegetables, servings/d	5	5	6	6	6
Red meat, servings/d	0.6	0.6	0.6	0.5	0.4
aHEI (excluding EPA + DHA)	42	43	45	47	50
Total energy intake, Kcal/d	1,728	1,761	1,769	1,748	1,700
HPFS					
Participants, No.	6,132	6,148	6,140	6,163	6,112
Age, y	63	64	64	64	65
¶BMI, kg/m^2	26.2	26.2	26.3	26.1	26.0
Caucasians, %	97	96	96	95	93
Current smokers, %	6	6	5	5	4
Pack years of smoking	13	12	12	11	10
Physical activity, MET-h/wk	32	33	35	35	39
Hypertension, %	35	36	37	38	39
Hypercholesterolemia, %	42	46	49	51	52
Current aspirin use, %	59	62	62	62	62

Table 2.1. (Continued) Age-standardized characteristics of participants according to cumulative average intake of EPA + DHA in 1998 (middle of follow-up)

	EPA + DHA (diet + supplements), Quintiles				
	Q1	Q2	Q3	Q4	Q5
Fish oil supplements, %	0	1	2	6	16
Dietary intake					
ALA, mg/d	1,113	1,111	1,110	1,102	1,122
EPA + DHA, mg/d	93	184	268	373	697
LA, g/d	11	11	11	11	11
Fruits and vegetables, servings/d	5	5	6	6	7
Red meat, servings/d	0.7	0.7	0.6	0.5	0.4
aHEI (excluding EPA + DHA)	43	44	46	48	51
Total energy intake, Kcal/d	1,982	2,015	2,011	1,951	1,933

All the values (except for age) are medians or percentages and are standardized to the age distribution of the study population

Abbreviations: MET-h, hours of metabolic equivalent tasks; aHEI, alternative healthy eating index; BMI, body mass index; ALA, α-linolenic acid; LA, linoleic acid.

[¶]BMI is calculated as weight in kilograms divided by height in meters squared.

[§]Current menopausal hormone use among postmenopausal women.

Table 2.2. Hazard ratios of intermediate and advanced AMD according to cumulative average intake of EPA and DHA

	Quintiles					
	Q1	Q2	Q3	Q4	Q5	p trend
Intermediate AMD						
EPA						
NHS						
Median (mg/d)	15	35	54	82	140	
Cases/person-years	236 /300,256	260 /297,224	229 /303,226	254 /306,019	230 /302,315	
Age-adjusted HR	1 (ref)	1.10	0.95	1.03	0.90	0.14
Multivariate HR (95% CI)	1 (ref)	1.06 (0.89,1.27)	0.92 (0.76,1.10)	1.00 (0.83,1.2)	0.88 (0.73,1.07)	0.13
HPFS						
Median (mg/d)	25	58	93	135	248	
Cases/person-years	80 /122,739	86 /123,084	68 /124,924	67 /121,765	79 /122,436	
Age-adjusted HR	1 (ref)	1.02	0.81	0.8	0.86	0.21
Multivariate HR (95% CI)	1 (ref)	1.01 (0.74,1.37)	0.80 (0.58,1.11)	0.84 (0.6,01.17)	0.90 (0.65,1.25)	0.47
Pooled	***1 (ref)***	***1.06 (0.91,1.23)***	***0.90 (0.76,1.05)***	***0.96 (0.82,1.13)***	***0.89 (0.75,1.05)***	***0.09***
DHA						
NHS						
Median (mg/d)	52	89	123	169	279	
Cases/person-years	259 /302,824	238 /297,361	249 /305,730	253 /300,047	210 /303,078	
Age-adjusted HR	1 (ref)	0.94	0.96	0.97	0.77	0.008
Multivariate HR (95% CI)	1 (ref)	0.93 (0.78,1.11)	0.94 (0.79,1.12)	0.95 (0.80,1.14)	0.76 (0.62,0.92)	0.008
HPFS						
Median (mg/d)	70	128	180	246	390	
Cases/person-years	84 /122,484	82 /124,126	68 /122,714	67 /121,959	79 /123,665	
Age-adjusted HR	1 (ref)	0.93	0.80	0.77	0.81	0.14
Multivariate HR (95% CI)	1 (ref)	0.91 (0.67,1.23)	0.80 (0.58,1.11)	0.80 (0.58,1.12)	0.85 (0.61,1.18)	0.35
Pooled	***1 (ref)***	***0.93 (0.80,1.08)***	***0.91 (0.78,1.07)***	***0.92 (0.79,1.07)***	***0.78 (0.66,0.92)***	***0.008***

Table 2.2. (Continued) Hazard ratios of intermediate and advanced AMD according to cumulative average intake of EPA and DHA

	Quintiles					
	Q1	Q2	Q3	Q4	Q5	p trend
EPA + DHA						
NHS						
Median (mg/d)	70	123	179	250	390	
Cases/person-years	252 /301,969	248 /299,460	228 /302,148	262 /302,831	219 /302,632	
Age-adjusted HR	1 (ref)	1	0.90	1.02	0.82	0.04
Multivariate HR (95% CI)	1 (ref)	0.98 (0.82,1.17)	0.88 (0.74,1.06)	1.00 (0.84,1.19)	0.80 (0.66,0.97)	0.03
HPFS						
Median (mg/d)	94	187	275	380	625	
Cases/person-years	80 /121,668	89 /124,756	71 /122,400	58 /122,937	82 /123,188	
Age-adjusted HR	1 (ref)	1.05	0.86	0.69	0.87	0.15
Multivariate HR (95% CI)	1 (ref)	1.03 (0.76,1.40)	0.86 (0.62,1.19)	0.73 (0.52,1.03)	0.92 (0.66,1.27)	0.38
Pooled	***1 (ref)***	***1.00 (0.86,1.17)***	***0.88 (0.75,1.04)***	***0.94 (0.80,1.10)***	***0.83 (0.71,0.98)***	***0.03***
EPA + DHA (supplement users excluded)						
NHS						
Median(mg/d)	66	118	170	233	355	
Cases/person-years	237 /286,521	234 /281,743	213 /281,446	232 /280,475	207 /275,902	
Age-adjusted HR	1 (ref)	1.01	0.91	0.98	0.86	0.09
Multivariate HR (95% CI)	1 (ref)	0.99 (0.82,1.19)	0.89 (0.74,1.08)	0.96 (0.80,1.16)	0.84 (0.69,1.03)	0.09
HPFS						
Median(mg/d)	90	180	260	358	564	
Cases/person-years	76 /114,609	83 /114,692	61 /111,874	55 /111,261	69 /109,760	
Age-adjusted HR	1 (ref)	1.04	0.81	0.71	0.81	0.07
Multivariate HR (95% CI)	1 (ref)	1.03 (0.75,1.41)	0.80 (0.57,1.13)	0.76 (0.53,1.08)	0.88 (0.63,1.25)	0.29
Pooled	***1 (ref)***	***1.01 (0.86,1.18)***	***0.88 (0.75,1.04)***	***0.92 (0.78,1.08)***	***0.86 (0.72,1.02)***	***0.04***

Table 2.2. (Continued) Hazard ratios of intermediate and advanced AMD according to cumulative average intake of EPA and DHA

	Quintiles					
	Q1	Q2	Q3	Q4	Q5	p trend
Advanced AMD						
EPA						
NHS						
Cases/person-years	182 /300,294	201 /297,277	198 /303,243	225 /306,041	204 /302,325	
Age-adjusted HR	1 (ref)	1.11	1.07	1.20	1.06	0.60
Multivariate HR (95% CI)	1 (ref)	1.07 (0.88,1.31)	1.05 (0.86,1.29)	1.21 (0.99,1.48)	1.11 (0.89,1.36)	0.29
HPFS						
Cases/person-years	80 /122,749	66 /123,111	64 /124,929	62 /121,771	74 /122,437	
Age-adjusted HR	1 (ref)	0.81	0.77	0.77	0.82	0.37
Multivariate HR (95% CI)	1 (ref)	0.84 (0.60,1.16)	0.82 (0.59,1.14)	0.88 (0.62,1.23)	0.99 (0.71,1.38)	0.70
Pooled	***1 (ref)***	***1.00 (0.84,1.19)***	***0.98 (0.83,1.17)***	***1.11 (0.94,1.32)***	***1.07 (0.89,1.28)***	***0.37***
DHA						
NHS						
Cases/person-years	185 /302,871	216 /297,373	205 /305,774	210 /300,073	194 /303,089	
Age-adjusted HR	1 (ref)	1.21	1.12	1.15	1.02	0.66
Multivariate HR (95% CI)	1 (ref)	1.20 (0.98,1.46)	1.12 (0.92,1.37)	1.17 (0.96,1.44)	1.06 (0.86,1.32)	0.90
HPFS						
Cases/person-years	84 /122,496	65 /124,148	71 /122,708	54 /121,976	72 /123,669	
Age-adjusted HR	1 (ref)	0.75	0.82	0.62	0.75	0.10
Multivariate HR (95% CI)	1 (ref)	0.76 (0.55,1.05)	0.87 (0.63,1.21)	0.71 (0.50,1.01)	0.89 (0.64,1.24)	0.67
Pooled	***1 (ref)***	***1.06 (0.90,1.26)***	***1.04 (0.88,1.24)***	***1.03 (0.86,1.23)***	***1.01 (0.84,1.21)***	***0.75***
EPA + DHA						
NHS						
Cases/person-years	177 /302,026	216 /299,479	205 /302,179	211 /302,856	201 /302,639	
Age-adjusted HR	1 (ref)	1.25	1.17	1.20	1.09	0.93
Multivariate HR (95% CI)	1 (ref)	1.23 (1.01,1.50)	1.17 (0.96,1.44)	1.22 (0.99,1.5)	1.15 (0.93,1.42)	0.49

Table 2.2. (Continued) Hazard ratios of intermediate and advanced AMD according to cumulative average intake of EPA and DHA

	Quintiles					
	Q1	Q2	Q3	Q4	Q5	p trend
HPFS						
Cases/person-years	78 /121,683	74 /124,775	62 /122,405	62 /122,938	70 /123,196	
Age-adjusted HR	1 (ref)	0.91	0.76	0.77	0.78	0.13
Multivariate HR (95% CI)	1 (ref)	0.93 (0.68,1.28)	0.82 (0.58,1.15)	0.88 (0.62,1.24)	0.93 (0.66,1.31)	0.77
Pooled	***1 (ref)***	***1.14 (0.96,1.35)***	***1.06 (0.89,1.27)***	***1.11 (0.93,1.33)***	***1.08 (0.90,1.29)***	***0.86***
EPA + DHA (supplement users excluded)						
NHS						
Cases/person-years	170 /286,565	189 /281,778	187 /281,460	195 /280,510	178 /275,921	
Age-adjusted HR	1 (ref)	1.15	1.14	1.17	1.06	0.82
Multivariate HR (95% CI)	1 (ref)	1.12 (0.91,1.38)	1.13 (0.91,1.39)	1.18 (0.95,1.45)	1.08 (0.87,1.35)	0.57
HPFS						
Cases/person-years	77 /114,618	64 /114,715	63 /111,870	52 /111,272	47 /109,775	
Age-adjusted HR	1 (ref)	0.81	0.81	0.68	0.56	0.002
Multivariate HR (95% CI)	1 (ref)	0.82 (0.59,1.15)	0.85 (0.61,1.20)	0.77 (0.53,1.10)	0.68 (0.46,0.99)	0.05
Pooled	***1 (ref)***	***1.03 (0.86,1.23)***	***1.04 (0.87,1.24)***	***1.05 (0.88,1.26)***	***0.96 (0.79,1.16)***	***0.29***

Multivariate models were adjusted for: age (continuous), race (Caucasians or not), BMI (<18.5, 18.5-23, 23-25, 25-30, 30-35, ≥ 35 kg/m^2), pack-years of smoking (never, 1-9, 10-24, 25-44, 45-64, ≥65y), physical activity (<3, 3-8.9, 9-17.9, 18-26.9, ≥27 MET-h/wk), current aspirin use (≥1 tablets/wk or none), history of hypertension and hypercholesterolemia, dietary variables including aHEI (excluding EPA and DHA), ALA, total energy intake (all in quintiles). In NHS, models were additionally adjusted for postmenopausal status and menopausal hormone use (never, current and past).

DHA and EPA included intake from diet and supplements unless otherwise specified.

Table 2.3. Pooled hazard ratios of intermediate and advanced AMD according to cumulative average intake of fish

	Servings/wk	Multivariate HR (95% CI)					
Fish[‡]	Median	Almost never	1-3 servings/mo	1 serving/wk	2-4 servings/wk	≥ 5 servings/wk	p trend
Intermediate AMD							
Canned tuna	0.70	1 (ref)	0.94 (0.83,1.07)	0.84 (0.73, 0.96)		0.68 (0.44,1.05)	0.005
Dark fish	0.20	1 (ref)	1.08 (0.91,1.27)	0.92 (0.74, 1.14)			0.74
Other fish[a]	0.58	1 (ref)	0.96 (0.85,1.09)	0.91 (0.79, 1.05)		0.91 (0.45,1.83)	0.25
Total fatty fish[b]	0.98	1 (ref)	0.92 (0.80,1.06)	0.95 (0.82,1.10)	0.79 (0.65,0.96)	0.61 (0.46,0.81)	<.001
Advanced AMD							
Canned tuna	0.70	1 (ref)	1.10 (0.96,1.26)	1.00 (0.86,1.16)		0.76 (0.46,1.23)	0.34
Dark fish	0.20	1 (ref)	1.04 (0.87,1.24)	0.98 (0.78,1.23)			0.97
Other fish[a]	0.58	1 (ref)	1.08 (0.94,1.23)	1.04 (0.89,1.22)		1.00 (0.44,2.24)	0.63
Total fatty fish[b]	0.98	1 (ref)	1.10 (0.94,1.29)	1.05 (0.90,1.24)	0.99 (0.80,1.22)	0.80 (0.59,1.08)	0.11

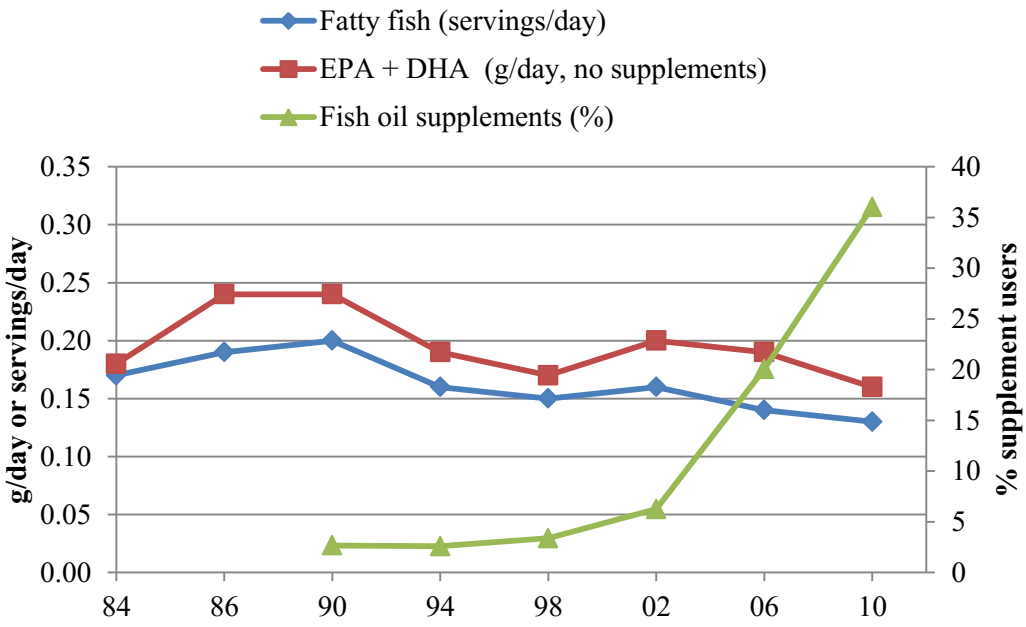
[‡] Multivariate models were adjusted for the same variable as in Table 2.

[a]Other fish were mainly white fish.

[b]Total fatty fish was the sum of canned tuna and dark fish. Finer intake categories were created to show detailed associations.

Figure 2.1. Time trend of age-adjusted mean intake of EPA + DHA, fatty fish and fish oil supplements

A. NHS



B. HPFS

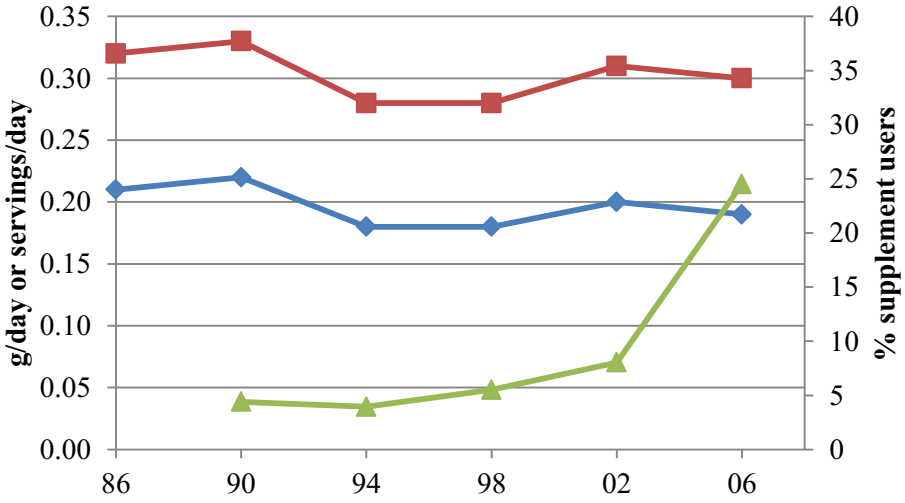
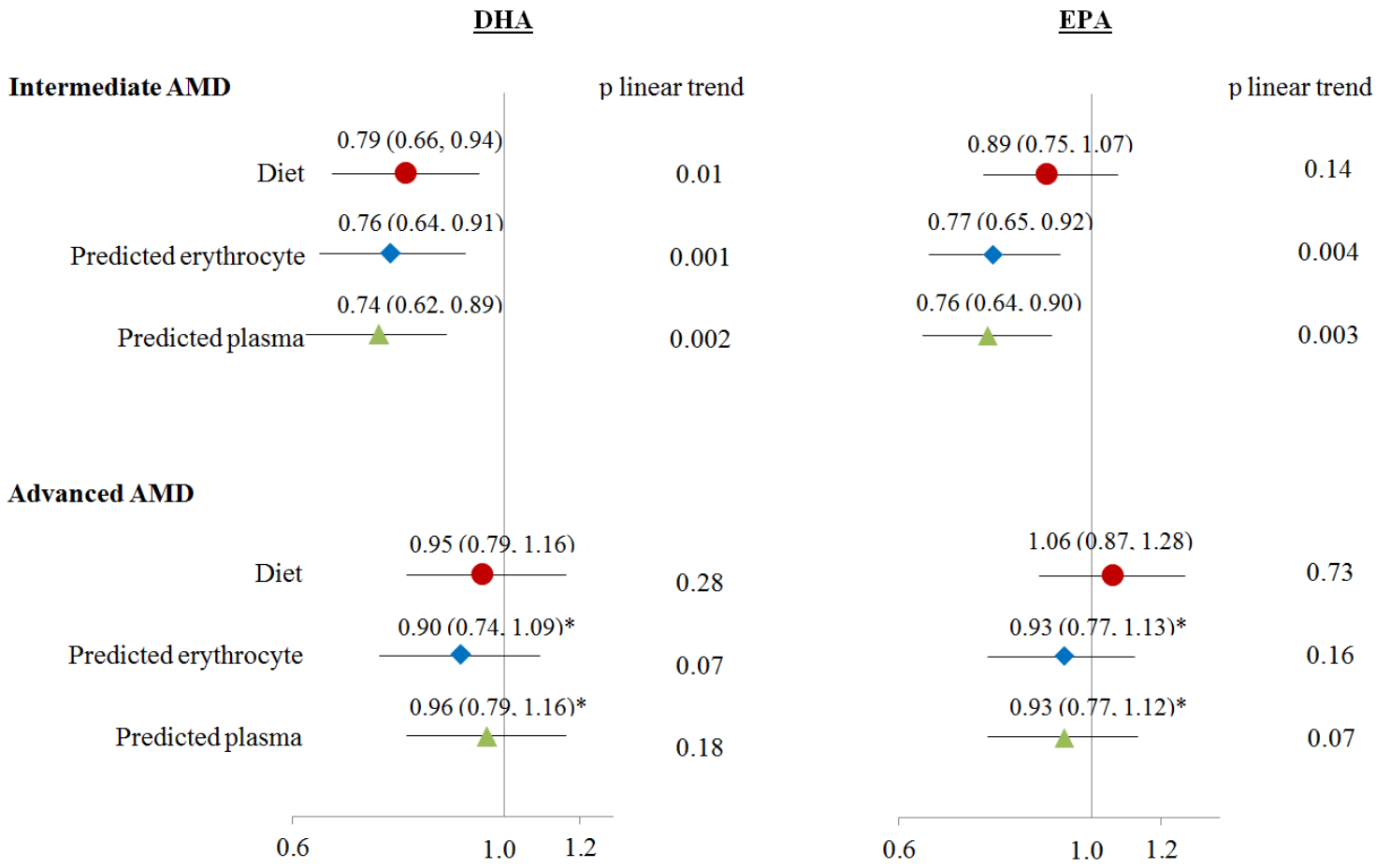


Figure 2.2. Pooled hazard ratios of AMD according to dietary intake, predicted erythrocyte and plasma scores of EPA and DHA among non-users of fish oil supplements



Multivariate-adjusted HR[§] comparing top to bottom quintiles

[§]Multivariate models were adjusted for the same variables as in the Table 2.2
*p for heterogeneity between the HRs from the NHS and HPFS was < 0.05
.

CHPATER III

DIETARY INTAKE OF α-LINOLENIC ACID AND RISK OF AGE-RELATED MACULAR DEGENERATION

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ABSTRACT

Background: Some observational studies suggested that marine omega3 fatty acids were protective for age-related macular degeneration (AMD). However, the role for the plant-derived shorter-chain omega3 fatty acid, α-linoleic acid (ALA), is unclear. European researchers reported that up to 40% of ALA can be present as *trans* forms.

Objectives: To evaluate the associations between intake of ALA and the intermediate and advanced AMD.

Design: 75,889 women from Nurses' Health Study and 38,961 men from Health Professionals Follow-Up Study were followed from 1984 to 2012 and 1986 to 2010, respectively. We assessed dietary intake by a validated food frequency questionnaire at baseline and every four years. During the follow-up, we confirmed 1,589 incident intermediate and 1,356 advanced AMD cases (primarily neovascular AMD) by medical record review.

Results: Higher intake of ALA was significantly associated with intermediate AMD before 2002 (comparing extreme quintiles, pooled HR = 1.36; 95% CI = 1.06 – 1.75; p trend = 0.008) but not after 2002 (pooled HR = 0.85; 95% CI = 0.64 – 1.13; p trend = 0.21), and p for interaction = 0.003. ALA intake was not associated with advanced AMD in either time period. By gas-liquid chromatography, we identified both *cis* ALA (mean, 0.13 ± 0.04%) and *trans* ALA isomers (mean, 0.05 ± 0.01%) in 395 erythrocyte samples collected in1989 -1990. In stepwise regression models, mayonnaise was the leading predictor of erythrocyte levels of *cis* ALA and one isomer of *trans* ALA. We also found *trans* ALA isomers in mayonnaise samples.

Conclusion: High intake of ALA was associated with an increased risk of intermediate AMD before 2002. This coincides with the same time period when *trans* ALA was found in

participants' blood and in mayonnaise, a primary food source of ALA. Whether *trans* ALA mediates this positive association warrants further studies.

I. Introduction

Age-related macular degeneration (AMD) is a chronic, progressive disease that can cause irreversible blindness.[1] According to the 2005-2008 US National Health and Nutrition Examination Survey (NHANES), the estimated prevalence in persons aged 40 years and older was 5.7% for early/intermediate AMD versus 0.8% for advanced AMD.[2] Because the number of AMD patients is expected to grow exponentially in the next few decades in the US and worldwide as populations ages,[3-5] the identification of potential strategies for primary prevention would have marked public health significance.

Marine long-chain omega3 (n3) fatty acids, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been associated with a lower risk of AMD in multiple observational studies.[6-10] However, epidemiologic research on the shorter-chain n3 fatty acid, α-linolenic acid (ALA), has yielded inconsistent findings. Some studies reported a positive association for intake of ALA[6,11] and vegetable fat foods[12-14] that contain ALA whereas others did not.[7,8,15,16] Additional studies of ALA's effects are needed because this plant-derived fatty acid accounts for at least 85% of total n3 fatty acid intake in the US[17] and, presumably even more, in parts of the world where access to fish is limited.

Trans ALA is formed during partial hydrogenation, deep frying and industrial deodorization[18,19] and is hypothesized to interfere with the crucial biological functions of *cis* ALA. *Trans* ALA has not received much attention in the US. However, European researchers have reported the presence of *trans* isomers of ALA in food such as vegetable oils,[18,20] low-calorie spreads[21] and infant formulas[22,23]. Up to 40% of ALA can be present as *trans* isomers.[19,20] *Trans* ALA also

occurs in human serum[24] and maternal milk[25,26]. Rats fed a diet high in *trans* ALA developed disturbed visual function and a decrease in *cis* DHA in the retina.[27,28]

We previously reported a positive association between intake of ALA and AMD.[6] With 16 years longer follow-up and 2,500 more AMD cases than our previous study, we aimed to evaluate this association in greater detail. Our secondary aim was to explore the role of *trans* ALA in the development of AMD.

II. Methods

Study Population

The Nurses' Health Study (NHS), initiated in 1976, is an ongoing prospective cohort that includes 121,700 US female registered nurses aged between 30 to 55 years at baseline. The Health Professionals Follow-up Study (HPFS), initiated in 1986, includes 51,529 US male health professionals aged between 40 and 75 years at baseline. Both cohorts are predominantly white. Participants are mailed biennial questionnaires about lifestyle factors and disease outcomes, and food frequency questionnaires (FFQ's) every four years to assess diet in the preceding years. The follow-up rates for both cohorts are high (>95%). The study protocol was approved by the Institutional Review Boards at the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health.

We restricted the study population to those who were ≥50 years old at baseline and then added participants to the analysis once they reached age 50 years. Participants were censored at age 90 years to alleviate the concern of lower ascertainment of AMD cases (NHS, n=15; HPFS, n=528). At baseline, we excluded participants who did not return the initial FFQ, left more than 70 food items blank in the FFQ, reported implausible dietary intake (<600 or >3500 Kcal/d for the NHS

and <800 or > 4200 Kcal/d for the HPFS), had prevalent AMD, cancer (except nonmelanoma skin cancer), diabetes and cardiovascular disease. To minimize detection bias, we also excluded participants who never reported an eye exam over the entire follow-up period and skipped the person-time during any two-year interval in which they did not report an eye exam. Results did not materially change in sensitivity analyses including intervals lacking an eye exam. After exclusions, a total of 75,889 women and 38,961 men contributed to the analysis.

AMD Ascertainment

Our case definition has been previously validated by comparison with retinal images and medical records.[29] When a participant reported a diagnosis of AMD on a biennial questionnaire, we requested informed consent to review his/her medical record and then contacted the participant's eye doctor to either complete a standardized questionnaire or to send us copies of ocular records to confirm the diagnosis. The questionnaire requested information from the medical record on the date of initial diagnosis, treatment received for AMD, clinical signs of AMD, best corrected visual acuity, and whether, in the opinion of the treating doctor, the visual acuity loss was due primarily to AMD. Photos and/or OCTs were also reviewed when available to confirm clinical AMD lesions. We excluded cases with only small hard drusen (drusen size< 63 μm diameter circle). We defined intermediate AMD as having at least one of the following signs in at least one eye: intermediate drusen (≥ 63 and <125 μm), pigment abnormalities, large drusen (≥ 125 μm) or any non-central geographic atrophy (GA). We defined neovascular AMD as having any of the following signs in at least one eye: RPE detachment, subretinal neovascular membrane, disciform scar, or history of treatment with laser, photodynamic, or anti-VEGF therapy for AMD. Central GA was defined as having a central geographic atrophy lesion involving the center of the macula in at least one eye. Advanced AMD included both neovascular

AMD and central GA. Additionally, all case definitions, except those recent neovascular AMD cases that had anti-VEGF therapies, included a visual acuity of 20/30 or worse due primarily to AMD. This magnitude of vision loss is not only of clinical significance, but also is severe enough to warrant medical attention so as to minimize potential detection bias arising from differential health consciousness. The person was used as the unit of analysis, and the worst eye was used for classification.

Dietary Assessment

We began follow up in 1986 for the HPFS and 1984 for the NHS, when the first comprehensive FFQ with 131 items was administered, and assessed dietary intake approximately every four years thereafter. Intake of ALA is from many foods but the major sources among FFQ items include 1) mayonnaise or other creamy salad dressing (1 tbs); 2) oil and vinegar dressing (e.g. Italian,1 tbs); 3) beef, pork or lamb as a main dish (4-6 oz); 4) margarine (1 pat); 5) other cheese (e.g. American, cheddar, 1 slice or 1 oz serving). These foods collectively accounted for 46% of ALA intake in the NHS and 38% in the HPFS at baseline. However, the total contribution from these foods decreased to 16% in 2010 in the NHS and 12% in 2006 in the HPFS. Walnut consumption (1 oz) was asked from the 1998 FFQ and onward, which became the top contributor to ALA intake (18% in the NHS and 17% in the HPFS). Low-fat mayonnaise consumption (1 tbs) was asked from the 1994 FFQ and onward. We started asking the intake of flax seed oil and flax seed (Yes or No) from the 2006 FFQ in the NHS and further inquired the frequency of intake for flax seed (1 Tbs) in the 2010 FFQ in both the NHS and HPFS. Participants were asked to report how often, on average over the past year, they had consumed each food item (9 possible responses ranging from "≤1 time per month" to "≥6 times/day"). The FFQs specifically inquired about the usual type of fat used for frying, sautéing and baking. It also inquired about the usual

brand and type of margarine using an open-ended question. Such information was taken into account when calculating ALA intake. The daily nutrient intake was calculated by multiplying the consumption frequency of each food by its nutrient content and then summing across all foods. The fatty acid composition of food fried, baked or sautéed at home was modified by the type of fat reported. The nutrient composition data were primarily based on the US Department of Agriculture Nutrient Database supplemented with information from manufacturers and published reports. We adjusted all the nutrient intakes for total energy using the residual method to reflect the composition of the diet.[30]

The reproducibility and validity of FFQs in measuring fat intake has been assessed in both cohorts. The Pearson correlations between energy-adjusted intake of different fatty acids from two 1-wk diet records and from the FFQ ranged from 0.48 to 0.73 in the NHS[31] and from 0.37 to 0.75 in the HPFS[32]. The Spearman correlation between the percentage of fat intake calculated from the FFQ and the proportion of that fat in the adipose tissue was 0.34 for ALA, 0.37 for linoleic acid, and 0.40 for *trans* fatty acids in the NHS[33] and 0.50 for polyunsaturated fatty acids, 0.47 for linoleic acid (LA) and 0.29 for *trans* fatty acids in the HPFS[34].

Measurement of *trans* ALA and *cis* ALA

To confirm the presence of *trans* ALA in food supplies and in human bodies, we measured *trans* ALA among erythrocyte samples (n=395) from the NHS participants by gas-liquid chromatography and also re-analyzed some existing chromatography of mayonnaise samples collected before and after 2000. The methods were described elsewhere.[35] Each *trans* ALA peak was identified by comparison with *trans* ALA standards (linolenic acid methyl ester isomer mix, purchased from Sigma-Aldrich) and the amount was expressed as the percentage of total peak

area. We identified four *trans* ALA isomers: *trans* ALA-A and -B have two *trans* double bonds while *trans* ALA-C and -D have one *trans* double bond. The concentration of erythrocyte *trans* ALA-A was extremely low and below the meaningful concentration (mean < 0.01%) and was thus not included in the analysis. The overall coefficients of variations (CV) for *trans* ALA-B, -C, and -D were 23.6%, 33.4% and 40.8%, respectively. Higher CVs for *trans* ALA-C and -D were likely due to the overlapping of those two peaks with 11t-Eicosenoic Acid (20:1n-9t) and 8c-Eicosenoic Acid (20:1n-12c), respectively. However, because the within-batch CV for each peak was <20% (an acceptable limit), we statistically accounted for the high overall CVs by recalibrating the concentration of each *trans* ALA isomer from all batches to its average batch according to a method outlined by Rosner et al.[36] Because *trans* ALA-C and -D peaks usually overlapped in chromatography, we combined those two in the analysis to reduce measurement error. We estimated the reproducibility of erythrocyte *trans* ALA by re-analyzing existing chromatography from an earlier within-person stability study where 40 postmenopausal women gave three blood samples over 2-3 years.[37] In that study, the intra-class correlation (ICC) for *cis* ALA was 0.52. The ICC we calculated was 0.53 for *trans* ALA-B, 0.56 for *trans* ALA-C and 0.43 for *trans* ALA-D, all indicating fair to good reproducibility.

To better understand the food determinants of physiologic *cis* ALA level, we used previously measured blood biomarker data among participants of nested case-control studies of cardiovascular disease in the NHS and HPFS. We included all the cases and controls because cases were not yet cases at the time of blood collection. The details on blood collection and measurements have been previously described.[38,39]

Statistical Analysis

Participants contributed person-time to the analysis from the return of the baseline questionnaire if over age 50 years at baseline or from reaching 50 years old to the confirmed diagnosis of AMD, death, loss to follow-up or the end of follow-up (05/31/2012 for the NHS and 01/31/2010 for the HPFS), whichever occurred first. To best represent long-term intake and minimize measurement error,[40] we calculated the cumulative average intake of ALA by averaging all available FFQs up to the start of each two-year risk interval. The cumulative average value was categorized into quintiles based on the distribution in each cohort. Likewise, we calculated the cumulative average intakes of foods and categorize them into pre-specified groups.

We used time-varying Cox proportional hazards models to estimate the hazard ratios (HR) and 95% confidence intervals (CI) associated with intermediate and advanced AMD, respectively. To control as finely as possible for confounding by age and calendar time and any possible two-way interactions between these two time scales, we stratified the model jointly by age in months at the start of follow-up and calendar year of the current questionnaire cycle. We controlled for established and probable risk factors including race, body mass index (BMI), pack-years of smoking, physical activity, aspirin use, history of hypertension, history of hypercholesterolemia, menopausal status and postmenopausal hormone use (in the NHS only), dietary variables including intakes of total energy intake, LA and DHA.

One way of generating *trans* ALA is by industrial partial hydrogenation.[21] From early 2000s there was an increasing public awareness of adverse health effects by *trans* fatty acids and food industries started to reduce or eliminate them.[41,42] Several studies found a downward trend of total *trans* intake[43] and plasma level[44] around that time. We thus chose 2002 as the cutoff to evaluate the differential association between intake of ALA and AMD in the pre-2002 and post-2002 eras due to a potential change in *trans* ALA content over time. We calculated the

cumulative average of ALA intake separately in each time period. We created interaction terms between the time-varying ALA variable and the binary indicator for time period and used a likelihood ratio test to test the significance of interaction by time comparing models with and without the interaction terms. A similar approach was used to test interactions between intake of ALA and pre-specified risk factors including age, smoking status and menopausal hormone use.

To better understand the food determinants of *cis* and *trans* ALA on a physiologic level, we used stepwise linear regressions to select foods that were significantly predictive of the biomarker measurements ($p < 0.05$). This method has been described previously.[45,46] Briefly, we used the average of food intake from the 1986 and 1990 FFQs in the NHS and from the 1990 and 1994 FFQs in the HPFS to reduce the within-person variation of intake and to correspond with the time of blood draw. We developed the linear regression model by the biomarker type in each cohort.

We performed the statistical analyses separately in each cohort using SAS 9.3 (SAS Institute, Cary, NC). To derive a pooled HR, we first combined the two cohorts and then applied a Cox proportional hazards model in the pooled data stratified by the cohort. Interpretation of the data was mainly based on pooled HRs unless otherwise specified. All hypothesis tests were two-sided and used an α level of 0.05.

III. Results

Study During 28 years of follow-up in the NHS and 24 years in the HPFS, we confirmed 2,219 incident AMD cases (1,209 intermediate + 1,010 advanced cases) in women and 726 (380 intermediate + 346 advanced cases) cases in men. The advanced AMD cases were predominantly neovascular AMD (>96%).

In 1998 (the middle of follow-up), participants at the highest cumulative average intake of ALA were likely to have a higher BMI and pack-years of smoking and were less likely to exercise and have hypercholesterolemia (Table 3.1). In terms of dietary intake, they were likely to have higher intakes of DHA, LA, *trans* fat and saturated fat, but overall a higher healthy eating score.

ALA intake was moderately correlated (average pearson r = 0.62) with LA intake, the omega6 analog fatty acid that shares many food sources with ALA, but weakly correlated with intake of food-sourced EPA (r = 0.10) or DHA (r = 0.06). During the follow-up, the age-adjusted intake of ALA has increased about 50% in the NHS from 1984 (median intake, 1.03 g/d) to 2010 (1.58 g/d), and 29% in the HPFS from 1986 (1.12 g/d) to 2006 (1.44 g/d). In contrast, the intake of food-sourced EPA + DHA did not appreciably change during the follow-up (data not shown).

In the pooled analysis between the NHS and HPFS, comparing extreme quintiles, intake of ALA had a statistically significant positive association with intermediate AMD (comparing extreme quintiles, HR = 1.31; 95% CI = 1.21 – 1.53; p trend <.001) and this association was essentially unaltered after further adjusting for LA or saturated, monounsaturated and *trans* fatty acids (Table 3.2). The multivariate-adjusted HR for ALA was in the positive direction in each cohort although was only statistically significant in the NHS. For the baseline 1986 FFQ in the HPFS, we have further separated intake of ALA by animal and plant sources (such information was not available in the NHS). The median baseline intake was 350mg g/d for animal-sourced ALA and 700 mg/d for plant-sourced ALA, and they were weakly negatively correlated (Pearson r = -0.22). Comparing extreme quintiles, plant-sourced ALA (adjusted HR = 1.54; 95% CI = 1.04 – 2.28; p trend = 0.03) was more strongly associated with intermediate AMD than the animal-sourced counterpart (adjusted HR = 1.17; 95% CI = 0.85 – 1.62; p trend = 0.22). When they were mutually adjusted for each other, the HR did not materially change for plant-sourced ALA

(adjusted HR = 1.60; 95% CI = 1.08 – 2.38; p trend = 0.02) nor for animal-sourced ALA (adjusted HR = 1.23; 95% CI = 0.89 – 1.70; p trend = 0.13). Comparing extreme quintiles, intake of LA also had a positive association with intermediate AMD (adjusted HR = 1.22; 95% CI = 1.05 – 1.42; p trend = 0.006); however, after further adjusting for ALA, the association was attenuated and no longer significant (adjusted HR = 1.06; 95% CI = 0.88 – 1.27; p trend = 0.50).

In contrast, we did not find any significant association between intake of ALA and advanced AMD after multivariate adjustment (Table 3.2), nor for LA (pooled HR for comparing extreme quintiles = 1.08; 95% CI = 0.91 – 1.28; p trend = 0.25).

The association between intake of ALA and intermediate AMD was only apparent among never smokers (pooled HR comparing extreme quintiles = 1.66; 95% CI = 1.23-2.22; p trend = 0.001) as compared with ever smokers (pooled HR comparing extreme quintiles = 1.07; 95% CI = 0.83-1.37; p trend = 0.001) (p for interaction = 0.10) (Supplementary Table 3.1). There was no significant interaction by age (Supplementary Table 3.2).

To understand whether the association with intermediate AMD was specific to ALA, we examined the intakes of several other correlated fatty acids including *cis* 18:1, *trans* 18:1, *cis* 18:2, *trans* 18:2, saturated fat, and total *trans* fatty acids. Comparing extreme quintiles, intakes of *cis* 18:1, *cis* 18:2, *trans* 18:2, and saturated fat each had a significant positive association with intermediate AMD (Supplementary Figure 3.1). However, all the associations were attenuated and no longer statistically significant after adjusting for ALA, whereas in all scenarios ALA persisted with a significant positive association (Supplementary Figure 3.1). On the other hand, intakes of *trans* 18:1 (p trend = 0.05), *trans* 18:2 (p trend = 0.04) and total *trans* fatty acids (p

trend = 0.03) had a suggestively significant association with advanced AMD after adjusting for ALA (Supplementary Figure 3.2).

We identified several *trans* ALA isomers in erythrocytes and mayonnaise (Figure 3.1). The amount of total *trans* ALA (mean, 0.05 ± 0.01%) was lower compared to *cis* ALA (mean, 0.13 ± 0.04 %) in erythrocytes (Supplementary Table 3.3). Among individual isomers of *trans* ALA, the correlation with *cis* ALA was much stronger for *trans* ALA-B (Pearson r=0.65) as compared with *trans* C / D (r=0.17) (Supplementary Table 3.4). Mayonnaise was the leading significant predictor for both *cis* ALA and *trans* ALA-B (Supplementary Table 3.3). Mayonnaise was also positively related to the plasma and erythrocyte level of *cis* ALA among previously measured samples (Supplementary Table 3.5). Intake of mayonnaise before 2002 was associated with the risk of intermediate AMD (HR for per 5 servings/week increase = 1.30; 95% CI = 1.07-1.57; p trend = 0.009) (Figure 3.2).

Although we were unable to quantify the intake of *trans* ALA because our nutrient database does not currently contain such information, we designed several exploratory analyses to evaluate whether *trans* ALA might be responsible for the positive association between ALA and intermediate AMD using the intake of total ALA, especially during the early follow-up period, as a proxy for *trans* ALA.

1. Stratified analysis by time period before and after 2002

We speculated that the intake of *trans* ALA would be higher prior to 2002 when *trans* fatty acids were more prevalent. Our own data also lend some support to this speculation. Comparing the representative mayonnaise sample collected before 2000 to the one after 2000, the percentage of *trans* ALA among total ALA (*cis* + *trans*) decreased from 11.8% to 5.0%. Comparatively, the

percentage of *trans* 18:2 among total 18:2 (*cis* + *trans*) decreased from 0.88% to 0.69%. Therefore, we hypothesize that the positive association between intake of ALA and intermediate AMD would be stronger prior to 2002 than after. Consistent with our hypothesis, comparing extreme quintiles, intake of ALA was positively associated with intermediate AMD before 2002 but not after 2002 and the interaction by time period was significant (p=0.003) (Table 3.3). There was no significant association between intake of ALA and advanced AMD in either time period. Mayonnaise and oil & vinegar salad dressing were both positively associated with intermediate AMD before 2002 but neither were after 2002, nor did any other major ALA-bearing foods (Figure 3.2). In a sensitivity analysis, we used 1998 instead of 2002 as the cutoff for the two time periods. The association between intake of ALA and intermediate AMD in each time period did not materially change; the HR comparing extreme quintiles was 1.36 (95% CI = 1.00 – 1.83; p trend = 0.02) before 1998 and 1.00 (95% CI = 0.78 – 1.26; p trend = 0.91) after 1998, but the p for interaction was slightly attenuated (p = 0.08).

2. Joint effect with intake of DHA and ALA

Informed by animal studies, this analysis was based on the hypothesis that *trans* ALA would adversely affect the development of AMD by conversion to *trans* DHA, which would interfere and compete with natural *cis* DHA for its crucial functions at the retina. Compared to the intake level at the top tertile of DHA but bottom tertile of ALA, intake at the bottom tertile of DHA and top tertile of ALA had a 46% (HR= 1.46; 95% CI=1.17 - 1.83) increased risk of intermediate AMD, but the interaction between ALA and DHA was not statistically significant (p=0.16). There was no apparent interaction between ALA and LA (p for interaction = 0.69).

3. Effect modification by menopausal hormone use

Although the conversion rate from ALA to DHA is generally considered low (~ 1%), some studies observed a 62% higher conversion rate in women taking an oral contraceptives and an increased activity of the desaturation/chain elongation pathway in postmenopausal women receiving hormone therapy.[47,48] Based on that, we hypothesized that in postmenopausal women, the association between intake of ALA and intermediate AMD would be stronger among current users of menopausal hormones than non-current users because more *trans* ALA could be converted to *trans* DHA. Consistent with our hypothesis, the HR for the association between intake of ALA and intermediate AMD among current users was 1.84 (95% CI=1.16 – 2.92; p trend=0.04; cases = 315) whereas the HR among non-current users was 1.17 (95% CI=0.89 – 1.54; p trend=0.17; cases = 789) and the p for interaction = 0.01. When we further restricted this analysis to the pre-2002 era, the HR's among current users was 2.20 (95% CI=1.27 – 3.82; p trend=0.03; cases = 235) and among non-current users was 1.12 (95% CI=0.76 – 1.63; p trend=0.35; cases = 380) with the p for interaction = 0.005.

IV. Discussion

In this large prospective study with 24-28 years of follow-up, high intake of ALA was associated with a modest increased risk of visually significant intermediate AMD but not with advanced AMD. However, only ALA intake in the pre-2002 era seemed harmful, possibly due to temporal changes in the composition of *cis* and *trans* isomers in dietary ALA sources. In accordance with European studies, our US-based study also identified the presence of *trans* ALA in human blood samples and in food (e.g. mayonnaise). Exploratory analyses suggest a role for *trans* ALA in the development of intermediate AMD.

Confounding is the greatest concern for the finding of a positive association between intake of ALA and intermediate AMD, because ALA intake in our cohorts was mainly derived from vegetable fat foods (e.g. mayonnaise, salad dressing, margarine), processed baked foods and red meat.[49] However, several analyses suggest that ALA is the primary causal factor. First, after further adjusting for other fatty acids primarily derived from unhealthy sources, including monounsaturated fat (mainly from red meat in our cohorts), saturated fat, and *trans* fatty acids, the result was essentially unchanged. We further found the specificity for the positive association with ALA among many correlated fatty acids. Finally, although red meat was associated with higher risk of AMD. [6,50] the finding that ALA from animal sources had a weaker association with intermediate AMD than ALA from vegetable sources reduces the likelihood of major confounding.

The association between intake of ALA and intermediate AMD seemed only apparent among never smokers. Smoking is known to increase the oxidative state of the body[51] and one may speculate that this oxidative environment would destroy ALA which is susceptible to oxidation due to its highly unsaturated nature. In our blood samples, pack-years of smoking was inversely related to plasma or erythrocyte level of ALA in both men and women (data not shown). Several other studies also suggested inverse association between smoking status and the blood level of long-chain n3 fatty acids.[52-54] However, an alternative explanation is that, since smoking is a strong risk factor for AMD,[29,55] the impact of ALA may be obscured for smoking-related AMD.

In accordance with European literature, we were able to confirm *trans* ALA in food and blood samples of US participants, and we also found that one isomer of *trans* ALA shared the same food, mayonnaise, with the *cis* ALA. Information on the *trans* ALA intake in the US is virtually non-existent likely because the intake of its precursor *cis* ALA is already much lower compared

to oleic acid and LA, the precursors to major *trans* fatty acids in the US diet. For example, according to NHANES 2009-2010 data, the mean intake is 1.50 g/d for ALA, 26 g/d for oleic acid and 15 g/d for LA.[56] However, studies suggested that ALA is 12-15 times more easily to be isomerized into *trans* forms than LA due to its more unsaturated nature and up to 40% of ALA can be present as *trans* isomers in some foods.[19,20] Based on a few US food samples, we crudely estimated the ratio of *trans* ALA to *cis* ALA was 1:7.5. Assuming that this ratio is generalizable to other *trans* ALA-containing foods, this translates into an intake of 0.2 g/d of *trans* ALA on the basis of a 1.5 g/d mean intake of ALA in the US population,[17] which is slightly lower but on the same magnitude as the estimated intake among Dutch and Scottish (0.5 - 0.7 g/d) and French participants (0.2 - 0.4 g/d).[24] Our study and the European study[24] both suggested a low level of erythrocyte *trans* ALA (~ 0.05% of total fatty acids) under a habitual diet. However, a study among 50 Indian participants with a high use (86%) of ALA-rich cooking oils - canola and mustard, and a high consumption of fried snacks and sweets (>3 days/wk) reported a markedly higher level of *trans* ALA in serum, adipose and cheek epithelium tissues (content ranging from 1.2-1.8%).[57] In European study in which 88 healthy men were randomized to receive a diet high (1.41 g/d) and low (0.06 g/d) in *trans* ALA for 6 weeks, the amount of *trans* ALA on plasma lipids was significantly increased compared to the control group.[24] This study further showed a statistically significant increase in LDL:HDL ratio in the high *trans* ALA group.[58] These findings suggest that body composition of *trans* ALA level is responsive to dietary intake.

Our hypothesis that *trans* ALA accounts for the association with intermediate AMD has some biological plausibility. Long-term feeding rats of a diet high in *trans* ALA severely disturbed visual function and also resulted in a significant increase in *trans* DHA and a decrease in *cis* DHA.[27,28] In humans, *trans* ALA is absorbed and incorporated into tissue lipids.[24] Small amounts

of *trans* EPA and DHA are present in human platelets.[59] These data suggest that *trans* ALA may affect the development of AMD through conversion to *trans* DHA, which may interfere with the function of natural *cis* DHA in the retina.

Data from our exploratory analyses are in line with the hypothesis that *trans* ALA is implicated in the development of intermediate AMD, assuming intake of total ALA is a proxy for *trans* ALA. First, ALA was only associated with higher risk of intermediate AMD during early follow-up, which can be explained by a higher content of *trans* ALA in the era of widespread industrial partial hydrogenation and less attention to the deodorization of vegetable oils. Second, we found a stronger positive association in postmenopausal women currently using exogenous hormones than those not. This may be explained by a higher biological conversion rate from *trans* ALA to *trans* DHA promoted by exogenous hormones. Although the endogenous conversion from ALA to DHA is limited in humans (0-4%),[60,61], the conversion rate was noted to be higher in women than men;[47,62] and it was 62% even greater in those using exogenous hormones.[47,48] Finally, stronger association was found in those with highest intake of ALA and lowest DHA compared to the vice versa. An explanation along the same line of biological conversion is that more *trans* DHA would be produced from a high intake of *trans* ALA which would have a greater adverse effect particularly when the intake level of *cis* DHA was low. However, an alternative explanation is that *cis* ALA would compete with *cis* DHA in incorporation into membranes. The second explanation does not seem to be supported by our data. When we accounted for dietary intake of DHA, an increasing plasma or erythrocyte level of ALA was significantly associated with a higher (as opposed to a lower) level of plasma or erythrocyte DHA in women (there was no statistically significant relation in men), which is more consistent with the hypothesis of conversion than competition, and higher conversion rate in women.

Only two[6,11] of previous studies[6-8,11-16] found a positive association between intake of ALA and AMD. Some other studies reported a positive association for vegetable fat foods and processed baked goods which contained ALA.[11-13] Unlike long-chain n3 fatty acids whose dietary intake mainly comes from fatty fish or fish oil supplements, ALA is obtained from many different foods. Measurement error arising from a single assessment of diet in all previous studies (except ours) may have obscured a modest association for ALA. However, studies that found a positive association generally had a larger number of cases and assessed baseline diet in the early 1990s, the era when intake of *trans* ALA was presumably higher. Interestingly, intake of ALA as well as mayonnaise before 1995 was also prospectively associated with early age-related lens opacities among 440 NHS participants.[63,64] It would be interesting to see whether ALA, possibly the *trans* isomers, is implicated in a common biological mechanism that underlies the pathogenesis of chronic eye diseases.

Several limitations of our study are worth noting. First, residual confounding cannot be ruled out due to the observational nature of our study. Second, measurement error of ALA intake by FFQs may lead to an underestimate of the true association. Finally, because some fatty acids overlap with *trans* ALA peaks in chromatography, more rigorous examination is desirable to confirm and quantify *trans* ALA in human tissues and food supply.

In summary, high intake of the shorter chain n3 fatty acid, ALA, is associated with a 36% higher risk of intermediate AMD before 2002. Although intake of ALA seems to be safer after 2002 in the US where industrial partial hydrogenation has been greatly reduced, further examination of *trans* ALA in current food supply may still be prudent, in light of FDA's efforts to eliminate industrially produced *trans* fatty acids from food supply. Partially isomerized sources of ALA may still contribute to risk of AMD in some countries where partially hydrogenated oils are

widely used or the quality of industrial deodorization of vegetable oils is suboptimal. Studies that could directly measure *trans* ALA are warranted to provide more definitive insights into the role of *trans* ALA in the development of AMD.

V. References

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VI. Tables and Figures

Table 3.1. Age-standardized characteristics of participants in the NHS and HPFS according to cumulative average intake of ALA in 1998 (the middle of follow-up)

	ALA, quintiles				
	Q1	Q2	Q3	Q4	Q5
NHS					
Participants, No.	13,257	13,246	13,264	13,258	13,245
Age, y, median	64	63	63	64	64
[¶]BMI, kg/m^2, median	26.1	26.6	26.8	27.0	27.1
Caucasians, %	96	98	98	98	96
Current smokers, %	10	9	10	10	12
Pack years of smoking, median	23	23	24	24	26
Physical activity, MET-h/wk, median	18	18	17	17	16
Hypertension, %	44	44	44	44	44
Hypercholesterolemia, %	59	58	57	56	54
Postmenopausal, %	94	94	94	94	94
[§]Current menopausal hormone use, %	44	45	46	45	45
Current aspirin use, %	47	49	49	49	47
Dietary intake, median					
ALA, mg/d	738	860	943	1,036	1,242
DHA, mg/d	133	137	140	139	143
LA, g/d	7.3	8.1	8.7	9.3	10.8
Trans fat, g/d	2.5	2.7	2.9	2.9	3.0
Saturated fat, g/d	17.7	19.0	19.7	20.4	21.3
Monounsaturated fat, g/d	19.2	20.8	21.7	22.5	23.7
Alternative healthy eating index	50.6	50.8	51.0	51.2	52.1
Total energy intake, kcal/d	1,695	1,743	1,770	1,770	1,732
HPFS					
Participants, No.	6,148	6,115	6,161	6,091	6,180
Age, y, median	64	64	64	64	64
[¶]BMI, kg/m^2, median	25.5	26.0	26.3	26.4	26.4
Caucasians, %	94	95	96	96	95
Current smokers, %	5	5	4	5	6
Pack years of smoking, median	11	11	11	12	13
Physical activity, MET-h/wk, median	35	35	35	35	34
Hypertension, %	38	38	37	36	37
Hypercholesterolemia, %	51	50	48	47	45
Current aspirin use, %	61	63	63	62	58

Table 3.1. (Continued) Age-standardized characteristics of participants in the NHS and HPFS according to cumulative average intake of ALA in 1998 (the middle of follow-up)

	ALA, quintiles				
	Q1	Q2	Q3	Q4	Q5
Dietary intake, median					
ALA, mg/d	840	987	1,085	1,196	1,445
DHA, mg/d	206	206	203	206	213
LA, g/d	9.3	10.2	10.8	11.6	13.2
Trans fat, g/d	2.8	3.1	3.3	3.4	3.4
Saturated fat, g/d	19.7	21.7	22.9	23.8	24.9
Monounsaturated fat, g/d	23.3	25.6	26.9	28.1	29.5
Alternative healthy eating index	54.0	53.9	53.6	53.8	55.3
Total energy intake, kcal/d	1,934	1,985	1,997	2,009	1,968

Abbreviations: MET-h, hours of metabolic equivalent tasks; BMI, body mass index; ALA, α-linolenic acid; DHA, Docosahexaenoic acid; LA, linoleic acid.

[¶]BMI is calculated as weight in kilograms divided by height in meters squared.

[§]Current menopausal hormone use among postmenopausal women.

Table 3.2. Hazard ratios of AMD according to quintiles of cumulative average intake of ALA

	Q1	Q2	Q3	Q4	Q5	P for trend
Intermediate AMD						
NHS						
Median (mg/d)	760	805	893	978	1,090	
Cases/person-years	211 / 300,584	237 / 301,701	203 / 302,772	273 / 301,809	285 / 302,175	
Age-adjusted model	1 (ref)	1.19 (0.99,1.43)	1.02 (0.84,1.24)	1.36 (1.14,1.63)	1.38 (1.15,1.64)	<.001
Multivariate model	1 (ref)	1.17 (0.97,1.41)	1.01 (0.83,1.22)	1.33 (1.11,1.60)	1.34 (1.12,1.61)	<.001
Multivariate model A	1 (ref)	1.21 (1.00,1.47)	1.05 (0.85,1.29)	1.38 (1.12,1.69)	1.31 (1.05,1.64)	0.02
HPFS						
Median (mg/d)	850	990	1,093	1,213	1,425	
Cases/person-years	74 / 122,869	76 / 123,263	67 / 122,309	75 / 123,512	88 / 122,996	
Age-adjusted model	1 (ref)	1.06 (0.77,1.47)	0.95 (0.68,1.32)	1.06 (0.77,1.47)	1.22 (0.90,1.67)	0.20
Multivariate model	1 (ref)	1.04 (0.76,1.44)	0.93 (0.67,1.30)	1.03 (0.75,1.43)	1.20 (0.88,1.64)	0.24
Multivariate model A	1 (ref)	1.03 (0.75,1.43)	0.92 (0.65,1.30)	1.01 (0.72,1.43)	1.18 (0.82,1.69)	0.37
Pooled (NHS+HPFS)						
Multivariate model	*1 (ref)*	*1.15 (0.97,1.34)*	*0.99 (0.84,1.17)*	*1.26 (1.07,1.47)*	*1.31 (1.12,1.53)*	*<.0001*
Multivariate model A	*1 (ref)*	*1.16 (0.99,1.37)*	*1.01 (0.85,1.21)*	*1.28 (1.07,1.52)*	*1.27 (1.05,1.54)*	*0.01*
Multivariate model B	*1 (ref)*	*1.17 (0.99,1.38)*	*1.02 (0.85,1.22)*	*1.28 (1.07,1.54)*	*1.28 (1.05,1.56)*	*0.01*
Advanced AMD						
NHS						
Median (mg/d)	760	805	893	978	1,090	
Cases/person-years	189 / 300,571	196 / 301,736	212 / 302,750	195 / 301,877	218 / 302,246	
Age-adjusted model	1 (ref)	1.10 (0.90,1.35)	1.20 (0.99,1.46)	1.09 (0.89,1.33)	1.19 (0.98,1.44)	0.14
Multivariate model	1 (ref)	1.07 (0.88,1.31)	1.15 (0.94,1.40)	1.04 (0.85,1.27)	1.12 (0.92,1.36)	0.40
Multivariate model A	1 (ref)	1.06 (0.86,1.30)	1.13 (0.91,1.40)	1.01 (0.81,1.27)	1.07 (0.84,1.36)	0.77
HPFS						
Median (mg/d)	850	990	1,093	1,213	1,425	
Cases/person-years	66 / 122,877	73 / 123,272	67 / 122,316	66 / 123,518	74 / 123,014	
Age-adjusted model	1 (ref)	1.15 (0.82,1.60)	1.05 (0.75,1.48)	1.02 (0.73,1.44)	1.14 (0.81,1.58)	0.66
Multivariate model	1 (ref)	1.11 (0.80,1.55)	1.02 (0.73,1.44)	0.97 (0.69,1.36)	1.08 (0.77,1.51)	0.88
Multivariate model A	1 (ref)	1.09 (0.78,1.53)	0.98 (0.69,1.40)	0.90 (0.63,1.31)	1.02 (0.69,1.49)	0.81

Table 3.2. (Continued) Hazard ratios of AMD according to quintiles of cumulative average intake of ALA

	Q1	Q2	Q3	Q4	Q5	P for trend
Pooled (NHS+HPFS)						
Multivariate model	*1 (ref)*	*1.09 (0.92,1.29)*	*1.12 (0.94,1.32)*	*1.02 (0.86,1.22)*	*1.11 (0.94,1.32)*	*0.40*
Multivariate model A	*1 (ref)*	*1.07 (0.90,1.28)*	*1.09 (0.91,1.31)*	*0.98 (0.81,1.19)*	*1.06 (0.86,1.30)*	*0.88*
Multivariate model B	*1 (ref)*	*1.06 (0.89,1.27)*	*1.08 (0.89,1.30)*	*0.97 (0.80,1.18)*	*1.05 (0.85,1.30)*	*0.92*

Multivariate model included: age (continuous), race (Caucasian or not), BMI (<18.5, 18.5-23, 23-25, 25-30, 30-35, ≥ 35 kg/m2), pack-years of smoking (never, 1-9, 10-24, 25-44, 45-64, ≥65y), physical activity (<3, 3-8.9, 9-17.9, 18-26.9, ≥27 MET-h/wk), current aspirin use (≥1 tablets/wk or none), history of hypertension and hypercholesterolemia, DHA (quintiles), total energy intake (quintiles). In the NHS, models were additionally adjusted for postmenopausal status and menopausal hormone use (never, current and past).
Multivariate model A = multivariate model + LA (quintiles)
Multivariate model B = multivariate model + LA + monounsaturated fat + saturated fat + *trans* fat (all in quintiles)

Table 3.3. Hazard ratios of AMD according to quintiles of cumulative average intake of ALA before and after 2002

	Q1	Q2	Q3	Q4	Q5	P for trend	P for interaction
Intermediate AMD							
Beginning - 2002							
NHS (cases n= 683)							
Median (mg/d)	748	865	957	1,060	1,242		
Multivariate model B	1 (ref)	1.18 (0.91,1.53)	1.00 (0.75,1.33)	1.36 (1.03,1.80)	1.33 (0.98,1.80)	0.05	
HPFS (cases n= 249)							
Median (mg/d)	830	970	1,075	1,197	1,410		
Multivariate model B	1 (ref)	1.05 (0.69,1.59)	0.90 (0.57,1.40)	1.16 (0.75,1.80)	1.50 (0.95,2.38)	0.06	
2002 - the end							
NHS (cases n= 499)							
Median (mg/d)	710	875	1,010	1,190	1,620		
Multivariate model B	1 (ref)	0.94 (0.71,1.24)	1.10 (0.83,1.45)	0.89 (0.66,1.20)	0.85 (0.62,1.17)	0.25	
HPFS (cases n= 124)							
Median (mg/d)	870	1,050	1,200	1,380	1,790		
Multivariate model B	1 (ref)	1.05 (0.60,1.83)	1.14 (0.66,1.99)	1.23 (0.70,2.17)	0.81 (0.43,1.54)	0.57	
Pooled (NHS + HPFS)							
Beginning - 2002	1 (ref)	1.13 (0.91,1.41)	0.96 (0.76,1.22)	1.30 (1.03,1.64)	1.36 (1.06,1.75)	0.008	
2002 - the end	1 (ref)	0.96 (0.75,1.23)	1.12 (0.87,1.43)	0.95 (0.73,1.24)	0.85 (0.64,1.13)	0.21	0.003
Advanced AMD							
Beginning - 2002							
NHS (cases n= 435)							
Median (mg/d)	748	865	957	1,060	1,242		
Multivariate model B	1 (ref)	1.17 (0.84,1.64)	1.44 (1.03,2.01)	1.10 (0.77,1.59)	1.12 (0.76,1.64)	0.91	
HPFS (cases n= 189)							
Median (mg/d)	830	970	1,075	1,197	1,410		
Multivariate model B	1 (ref)	1.01 (0.64,1.60)	1.01 (0.62,1.63)	0.97 (0.58,1.59)	1.03 (0.60,1.79)	0.96	
2002 - the end							
NHS (cases n= 546)							
Median (mg/d)	710	875	1,010	1,190	1,620		
Multivariate model B	1 (ref)	0.78 (0.60,1.02)	0.93 (0.71,1.22)	1.07 (0.82,1.40)	0.82 (0.60,1.11)	0.54	

Table 3.3. (Continued) Hazard ratios of AMD according to quintiles of cumulative average intake of ALA before and after 2002

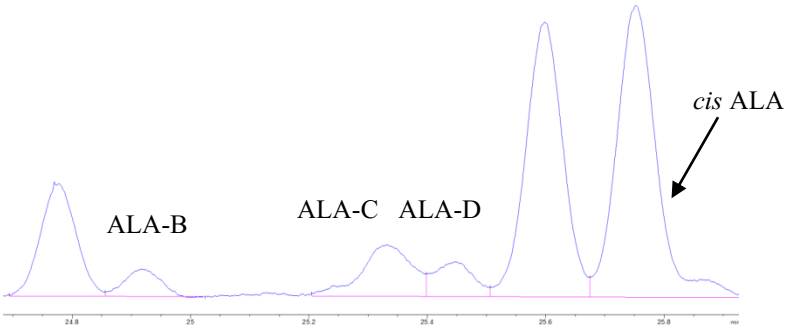
	Q1	Q2	Q3	Q4	Q5	P for trend	P for interaction
HPFS (cases n=147)							
Median (mg/d)	870	1,050	1,200	1,380	1,790		
Multivariate model B	1 (ref)	0.69 (0.39,1.21)	1.21 (0.73,1.99)	0.83 (0.48,1.44)	0.95 (0.55,1.65)	0.93	
Pooled (NHS + HPFS)							
Beginning - 2002	1 (ref)	1.11 (0.85,1.46)	1.28 (0.97,1.68)	1.05 (0.78,1.41)	1.09 (0.79,1.49)	0.96	
2002 - the end	1 (ref)	0.76 (0.59,0.96)	0.98 (0.78,1.24)	1.01 (0.79,1.29)	0.84 (0.65,1.10)	0.59	0.14

Multivariate model B was the same as in the Table 3.2

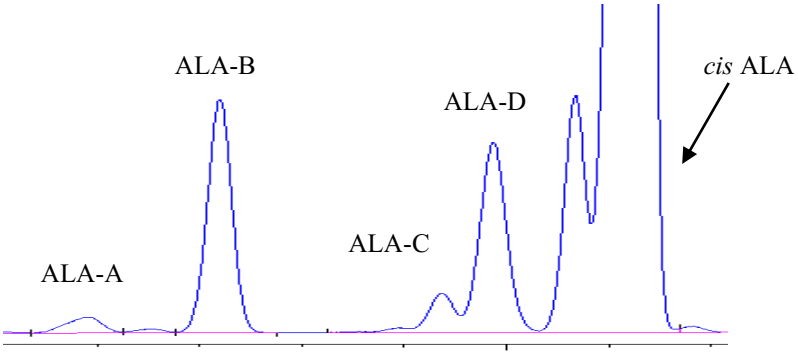
The number of cases does not add up to the number in the Table 2 because participants missing ALA intake in 2002 were excluded from the analysis of post-2002 era.

Figure 3.1. Partial gas-liquid chromatography of *trans* and *cis* ALA isomers

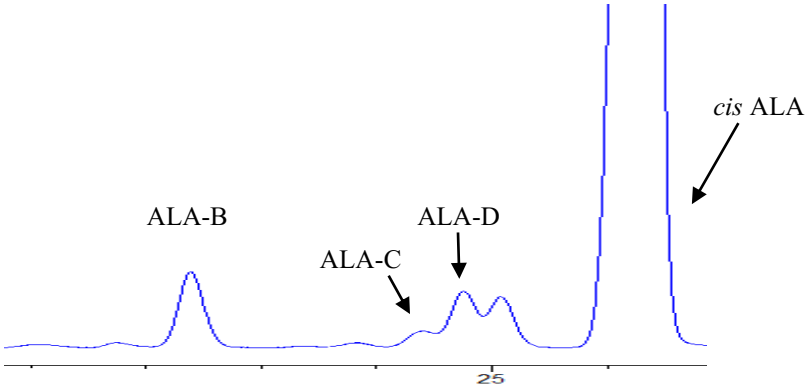
A. Erythrocyte (collected between 1989 and 1990)



B. Mayonnaise (samples collected before 2000)

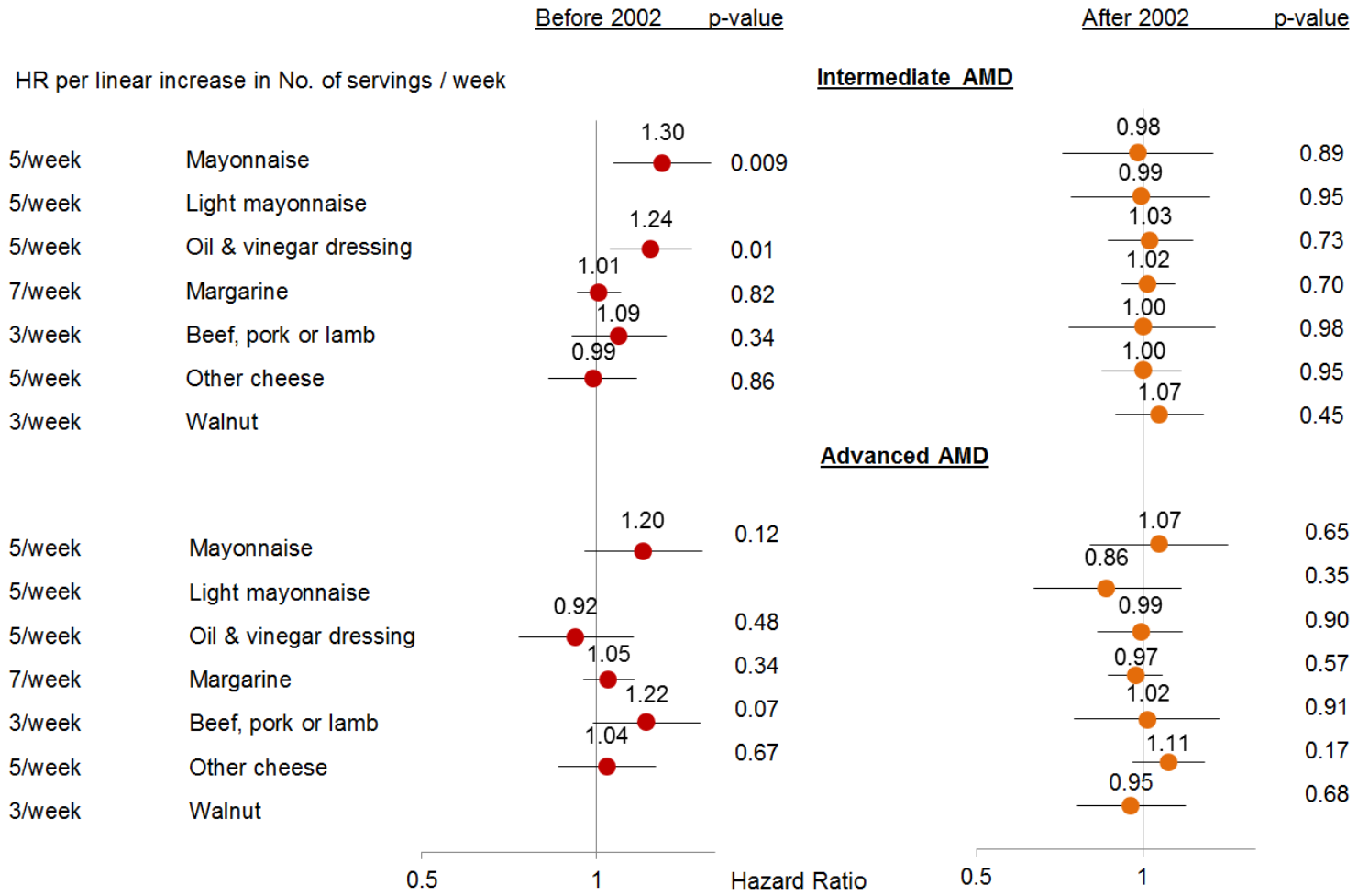


C. Mayonnaise (samples collected after 2000)



Trans ALA – A and – B denotes *trans* ALA with two *trans* bonds; ALA – C and – D denotes *trans* ALA with one *trans* bond. The level of *trans* ALA-A was extremely low and cannot always be identified in samples

Figure 3.2. The associations of primary ALA-containing foods with intermediate and advanced AMD



Multivariate model included: age (continuous), race (Caucasians or not), BMI (<18.5, 18.5-23, 23-25, 25-30, 30-35, ≥ 35 kg/m2), pack-years of smoking (never, 1-9, 10-24, 25-44, 45-64, ≥65y), physical activity (<3, 3-8.9, 9-17.9, 18-26.9, ≥27 MET-h/wk), current aspirin use (≥1 tablets/wk or none), history of hypertension, history of hypercholesterolemia, and total calories (quintiles). In the NHS, models were additionally adjusted for postmenopausal status and menopausal hormone use (never, current and past).

Supplementary Tables

Supplementary Table 1.1. β-coefficients (standard error) for plasma carotenoid[1] multivariate linear regression models among all participants (*n* = 4058-4177) (adapted from Hendrickson et al, *Nutrients* 2013)

Food[2]	α-carot.[3]	β-carot.[4]	β-crypto.[5]	Lutein/ zeax.[6]	Lycop.[7]	Food β-carot.[8]
Apples or pears, fresh			0.123 (0.024)			
Bananas	0.199 (0.032)					
Broccoli		0.311 (0.057)		0.204 (0.037)		0.312 (0.058)
Cantaloupe		0.360 (0.078)				0.325 (0.080)
Carrots, cooked	0.427 (0.071)					
Carrots, raw	0.675 (0.029)	0.341 (0.032)	0.095 (0.024)	0.063 (0.020)		0.339 (0.032)
Corn			-0.182 (0.067)			
Eggplant/zucchini/other summer squash				0.161 (0.059)		
Eggs				0.116 (0.028)		
Grape fruit						0.104 (0.052)
Juice, orange			0.281 (0.016)	0.097 (0.013)		
Juice, tomato					0.249 (0.055)	
Kale, mustard, or chard greens		0.495 (0.225)		0.308 (0.140)		
Lettuce, romaine or leaf		0.164 (0.034)		0.190 (0.021)		0.168 (0.035)
Oranges			0.442 (0.032)	0.060 (0.024)		
Peaches, apricots, or plums			0.251 (0.041)			
Peas or lima beans		-0.225 (0.085)				-0.215 (0.086)
Pizza		-0.377 (0.148)			0.608 (0.107)	-0.368 (0.149)
Popcorn				0.060 (0.018)		
Prunes		0.096 (0.047)	0.117 (0.037)			0.095 (0.047)
Spinach, cooked				0.265 (0.085)		
Spinach, raw				0.220 (0.076)		

Supplementary Table 1.1.(Continued) β-coefficients (standard error) for plasma carotenoid[1] multivariate linear regression models among all participants (*n* = 4058-4177) (adapted from Hendrickson et al, *Nutrients* 2013)

Food[2]	α-carot.[3]	β-carot.[4]	β-crypto.[5]	Lutein/ zeax.[6]	Lycop.[7]	Food β-carot.[8]
Supplemental β-carotene, 1990		0.133 (0.017)				
Tomato sauce					0.602 (0.061)	
Tomatoes	-0.061 (0.031)				0.121 (0.024)	
Yams or sweet potatoes		0.574 (0.174)				0.606 (0.174)

[1]Plasma carotenoid concentrations were natural log transformed and adjusted for age, case-control status, body mass index, plasma cholesterol, menopausal status, and hormone therapy use by the residual method

[2]Foods (servings/day, milligrams/day for supplemental β-carotene) selected by stepwise selection from all foods contributing ⩾ 0.5% to intake of the relevant carotenoid in the full cohort with 0.10 significance level to enter and 0.05 significance level to stay

[3]Intercept = 3.99, β (SE) for total energy intake = -0.0000710 (0.0000206), model adjusted R^2 = 0.14

[4]Intercept = 5.35, β (SE) for total energy intake = -0.0000670 (0.0000233), model adjusted R^2 = 0.09

[5]Intercept = 4.11, β (SE) for total energy intake = -0.0000716 (0.0000181), model adjusted R^2 = 0.15

[6]Intercept = 5.03, β (SE) for total energy intake = -0.0000702 (0.0000142), model adjusted R^2 = 0.09

[7]Intercept = 5.88, β (SE) for total energy intake = -0.0000608 (0.0000162), model adjusted R^2 = 0.05

[8]Intercept = 5.37, β (SE) for total energy intake = -0.0000676 (0.0000235), model adjusted R^2 = 0.08

Supplementary Table 1.2. Hazard ratios of AMD according to quintiles of predicted plasma carotenoid scores and calculated intakes in the NHS

	Advanced AMD						Intermediate AMD			
	Predicted plasma level				¶Calculated intake		Predicted plasma level			¶Calculated intake
Quintiles (median, µg/L)	Person-years	Cases	Age-adjusted HR	Multivariate HR (95% CI)	Quintiles (median, ug)	Multivariate HR (95% CI)	Cases	Age-adjusted HR	Multivariate HR (95% CI)	Multivariate HR (95% CI)
Lutein/Zeaxanthin										
Q1 (152)	221,930	175	1 (Ref.)	1 (Ref.)	1,408	1 (Ref.)	206	1 (Ref.)	1 (Ref.)	1 (Ref.)
Q2 (161)	222,331	151	0.83	0.83 (0.66,1.03)	2,098	0.84 (0.67,1.04)	198	0.93	0.96 (0.78,1.16)	0.82 (0.67,1.00)
Q3 (169)	222,557	175	0.93	0.92 (0.74,1.14)	2,680	0.78 (0.63,0.98)	192	0.87	0.91 (0.74,1.11)	0.91 (0.74,1.11)
Q4 (179)	222,865	157	0.83	0.81 (0.64,1.01)	3,389	0.72 (0.57,0.91)	188	0.84	0.89 (0.72,1.09)	0.93 (0.76,1.14)
Q5 (198)	222,585	115	0.60	0.59 (0.46,0.76)	4,834	0.68 (0.54,0.87)	199	0.88	0.94 (0.76,1.16)	0.90 (0.72,1.11)
p for trend			<.0001	<.0001		0.003		0.16	0.53	0.73
β-cryptoxanthin										
Q1 (59)	221,975	150	1 (Ref.)	1 (Ref.)	80	1 (Ref.)	187	1 (Ref.)	1 (Ref.)	1 (Ref.)
Q2 (65)	222,325	158	0.92	0.98 (0.78,1.23)	128	0.79 (0.62,0.99)	170	0.81	0.86 (0.70,1.06)	0.96 (0.78,1.18)
Q3 (72)	222,623	151	0.80	0.87 (0.69,1.10)	171	0.85 (0.68,1.07)	214	0.94	1.02 (0.84,1.25)	1.01 (0.82,1.24)
Q4 (89)	222,625	172	0.84	0.94 (0.75,1.17)	218	0.82 (0.66,1.03)	204	0.83	0.93 (0.76,1.14)	0.93 (0.76,1.15)
Q5 (93)	222,720	142	0.64	0.74 (0.58,0.93)	297	0.70 (0.55,0.88)	208	0.79	0.92 (0.75,1.13)	0.98 (0.79,1.20)
p for trend			0.0001	0.01		0.009		0.04	0.66	0.79
Lycopene										
Q1 (354)	222,439	203	1 (Ref.)	1 (Ref.)	3,322	1 (Ref.)	264	1 (Ref.)	1 (Ref.)	1 (Ref.)
Q2 (370)	222,454	173	1.03	1.01 (0.82,1.24)	4,693	1.05 (0.85,1.29)	205	0.92	0.92 (0.77,1.11)	1.01 (0.84,1.21)
Q3 (383)	222,524	147	1.01	0.98 (0.79,1.21)	5,814	1.03 (0.84,1.28)	190	0.97	0.97 (0.80,1.17)	0.93 (0.76,1.13)
Q4 (400)	222,622	124	0.95	0.91 (0.72,1.14)	7,205	0.76 (0.60,0.97)	158	0.88	0.88 (0.72,1.08)	1.01 (0.83,1.23)
Q5 (438)	222,229	126	1.10	1.04 (0.82,1.30)	9,909	1.04 (0.83,1.29)	166	1.04	1.03 (0.84,1.25)	1.05 (0.86,1.28)
p for trend			0.57	0.99		0.60		0.80	0.88	0.61

Supplementary Table 1.2. (Continued) Hazard ratios of AMD according to quintiles of predicted plasma carotenoid scores and calculated intakes in the NHS

α-carotene										
Q1 (51)	221,588	163	1 (Ref.)	1 (Ref.)	323	1 (Ref.)	191	1 (Ref.)	1 (Ref.)	1 (Ref.)
Q2 (55)	222,435	174	0.93	0.99 (0.79,1.22)	507	0.72 (0.58,0.91)	190	0.88	0.92 (0.75,1.13)	1.00 (0.81,1.23)
Q3 (58)	222,649	156	0.76	0.83 (0.66,1.04)	681	0.81 (0.65,1.01)	204	0.87	0.94 (0.77,1.16)	1.07 (0.87,1.33)
Q4 (65)	222,787	154	0.74	0.82 (0.65,1.03)	932	0.81 (0.65,1.01)	184	0.77	0.85 (0.69,1.05)	1.18 (0.96,1.45)
Q5 (79)	222,810	126	0.57	0.64 (0.50,0.82)	1,429	0.61 (0.48,0.77)	214	0.84	0.96 (0.78,1.18)	1.05 (0.85,1.30)
p for trend			<.0001	0.0001		0.0007		0.12	0.80	0.51
β-carotene										
Q1 (198)	221,515	145	1 (Ref.)	1 (Ref.)	2,278	1 (Ref.)	175	1 (Ref.)	1 (Ref.)	1 (Ref.)
Q2 (215)	222,388	167	1.00	1.01 (0.81,1.27)	3,312	1.07 (0.85,1.34)	192	0.95	0.97 (0.79,1.20)	1.05 (0.85,1.29)
Q3 (231)	222,589	164	0.92	0.96 (0.76,1.21)	4,220	1.07 (0.84,1.34)	210	0.98	1.03 (0.84,1.27)	1.12 (0.90,1.38)
Q4 (254)	222,858	139	0.74	0.78 (0.61,1.00)	5,353	0.84 (0.65,1.08)	190	0.85	0.92 (0.74,1.15)	1.10 (0.89,1.36)
Q5 (315)	222,917	158	0.83	0.88 (0.69,1.13)	7,584	0.86 (0.66,1.11)	216	0.94	1.04 (0.83,1.29)	1.02 (0.81,1.28)
p for trend			0.02	0.15		0.05		0.49	0.74	0.97
β-carotene from food										
Q1 (200)	214,856	151	1 (Ref.)	1 (Ref.)	2,151	1 (Ref.)	170	1 (Ref.)	1 (Ref.)	1 (Ref.)
Q2 (215)	214,310	160	0.91	0.90 (0.72,1.13)	3,092	0.98 (0.78,1.24)	179	0.91	0.93 (0.75,1.16)	0.84 (0.68,1.04)
Q3 (229)	212,920	147	0.78	0.78 (0.61,0.99)	3,913	0.91 (0.72,1.15)	191	0.91	0.96 (0.77,1.19)	0.98 (0.80,1.21)
Q4 (248)	211,231	151	0.78	0.78 (0.61,0.99)	4,907	0.78 (0.61,0.99)	185	0.86	0.94 (0.75,1.17)	0.97 (0.78,1.20)
Q5 (289)	208,284	135	0.66	0.66 (0.51,0.85)	6,759	0.66 (0.51,0.85)	205	0.91	1.02 (0.81,1.28)	0.91 (0.73,1.14)
p for trend			0.0003	0.001		0.0002		0.44	0.69	0.80
Total carotene from food										
Q1 (253)	214,758	152	1 (Ref.)	1 (Ref.)	2,668	1 (Ref.)	175	1 (Ref.)	1 (Ref.)	1 (Ref.)
Q2 (271)	214,258	170	0.94	0.94 (0.75,1.18)	3,865	1.05 (0.83,1.31)	174	0.84	0.87 (0.70,1.08)	0.90 (0.73,1.12)
Q3 (289)	213,028	140	0.73	0.73 (0.58,0.93)	4,927	0.91 (0.72,1.15)	201	0.92	0.98 (0.79,1.21)	1.08 (0.87,1.32)
Q4 (313)	210,987	146	0.73	0.74 (0.58,0.95)	6,274	0.75 (0.59,0.96)	175	0.78	0.85 (0.68,1.06)	0.95 (0.77,1.18)
Q5 (366)	208,570	136	0.65	0.66 (0.51,0.86)	8,855	0.70 (0.54,0.90)	205	0.87	0.98 (0.79,1.23)	0.99 (0.79,1.23)
p for trend			<.0001	0.001		0.0003		0.29	0.83	0.95

Supplementary Table 1.2. (Continued) Hazard ratios of AMD according to quintiles of predicted plasma carotenoid scores and calculated intakes in the NHS

[§]Total carotenoid index									
Q1 (9)	222,083	174	1 (Ref.)	1 (Ref.)	8	1 (Ref.)	218	1 (Ref.)	1 (Ref.)	1 (Ref.)
Q2 (12)	221,393	172	0.95	0.97 (0.78,1.20)	12	0.89 (0.71,1.11)	166	0.73	0.76 (0.62,0.93)	0.98 (0.80,1.21)
Q3 (15)	234,719	160	0.79	0.81 (0.65,1.01)	15	0.75 (0.60,0.94)	214	0.84	0.90 (0.74,1.09)	1.00 (0.81,1.22)
Q4 (18)	208,544	137	0.74	0.77 (0.61,0.97)	18	0.77 (0.61,0.96)	177	0.77	0.84 (0.68,1.03)	1.08 (0.88,1.33)
Q5 (21)	225,528	130	0.64	0.67 (0.53,0.86)	22	0.69 (0.55,0.87)	208	0.81	0.92 (0.75,1.13)	1.03 (0.83,1.26)
p for trend			<.0001	0.0003		0.001		0.09	0.76	0.60

Multivariate models were adjusted for: age (continuous), BMI (<18.5, 18.5-23, 23-25, 25-30, 30-35, ≥ 35 kg/m2), pack-years of smoking (never, 1-9, 10-24, 25-44, 45-64, ≥65y), physical activity (<3, 3-8.9, 9-17.9, 18-26.9, ≥27 MET-h/wk), current aspirin use (≥1 tablets/wk or none), history of hypertension, diabetes and cardiovascular diseases, postmenopausal status and menopausal hormone use (never, current and past), dietary variables including aHEI (excluding fruits and vegetables), alcohol intake, DHA and ALA (all in quintiles).

[§] Total carotenoid index was created by summing the quintile score of each carotenoid.

[¶] Models were additionally adjusted for total calorie intake (in quintiles).

Supplementary Table 1.3. Hazard ratios of AMD according to quintiles of predicted plasma carotenoid scores and calculated intakes in the HPFS

	Advanced AMD						Intermediate AMD			
	Predicted plasma level				Calculated intake		Predicted plasma level			Calculated intake
Quintiles (median, µg/L)	Person-years	Cases	Age-adjusted HR	Multivariate HR (95% CI)	Quintiles (median, ug)	Multivariate HR (95% CI)	Cases	Age-adjusted HR	Multivariate HR (95% CI)	Multivariate HR (95% CI)
Lutein/Zeaxanthin										
Q1 (151)	98,632	78	1(Ref.)	1(Ref.)	1,511	1(Ref.)	72	1(Ref.)	1(Ref.)	1(Ref.)
Q2 (161)	99,238	65	0.76	0.81 (0.58,1.13)	2,313	1.05 (0.75,1.47)	72	0.94	1.01 (0.72,1.40)	1.27 (0.92,1.76)
Q3 (169)	99,631	79	0.92	0.98 (0.71,1.35)	3,012	1.06 (0.75,1.49)	79	1.00	1.07 (0.77,1.48)	1.13 (0.81,1.58)
Q4 (180)	99,764	73	0.82	0.88 (0.63,1.23)	3,864	1.06 (0.75,1.50)	81	0.98	1.04 (0.75,1.44)	1.06 (0.75,1.50)
Q5 (203)	99,651	50	0.53	0.59 (0.41,0.86)	5,629	1.08 (0.75,1.55)	74	0.86	0.92 (0.66,1.30)	1.20 (0.84,1.70)
p for trend			0.001	0.01		0.71		0.41	0.62	0.65
β-cryptoxanthin										
Q1 (58)	98,452	67	1(Ref.)	1(Ref.)	82	1(Ref.)	71	1(Ref.)	1(Ref.)	1(Ref.)
Q2 (65)	99,347	63	0.80	0.87 (0.61,1.23)	141	0.94 (0.67,1.34)	69	0.84	0.90 (0.64,1.26)	0.98 (0.71,1.36)
Q3 (72)	99,481	72	0.78	0.86 (0.61,1.21)	194	0.90 (0.63,1.26)	82	0.86	0.93 (0.67,1.29)	0.90 (0.65,1.25)
Q4 (80)	99,868	74	0.72	0.82 (0.58,1.15)	253	0.88 (0.62,1.25)	83	0.77	0.87 (0.62,1.20)	0.83 (0.59,1.16)
Q5 (99)	99,767	69	0.62	0.73 (0.51,1.04)	358	0.75 (0.52,1.07)	73	0.62	0.70 (0.50,0.99)	0.72 (0.51,1.01)
p for trend			0.006	0.09		0.10		0.004	0.04	0.03
Lycopene										
Q1 (346)	99,214	115	1(Ref.)	1(Ref.)	3,259	1(Ref.)	105	1(Ref.)	1(Ref.)	1(Ref.)
Q2 (364)	99,328	82	0.91	0.94 (0.70,1.25)	5,079	0.96 (0.71,1.29)	79	0.96	1.01 (0.75,1.36)	0.92 (0.68,1.24)
Q3 (379)	99,617	61	0.81	0.84 (0.61,1.15)	6,609	0.84 (0.60,1.16)	74	1.07	1.13 (0.83,1.53)	1.11 (0.82,1.50)
Q4 (400)	99,563	47	0.71	0.74 (0.52,1.04)	8,599	0.79 (0.56,1.12)	65	1.08	1.13 (0.83,1.55)	1.13 (0.82,1.55)
Q5 (450)	99,195	40	0.69	0.70 (0.49,1.02)	12,589	0.93 (0.66,1.30)	55	1.04	1.07 (0.76,1.49)	1.04 (0.75,1.45)
p for trend			0.02	0.03		0.46		0.65	0.58	0.56

Supplementary Table 1.3. (Continued) Hazard ratios of AMD according to quintiles of predicted plasma carotenoid scores and calculated intakes in the HPFS

α-carotene										
Q1 (50)	98,248	60	1(Ref.)	1(Ref.)	345	1(Ref.)	77	1(Ref.)	1(Ref.)	1(Ref.)
Q2 (54)	99,174	67	0.94	1.00 (0.70,1.43)	551	1.02 (0.73,1.43)	59	0.67	0.75 (0.53,1.06)	0.81 (0.58,1.13)
Q3 (58)	99,716	79	1.03	1.16 (0.82,1.64)	753	0.84 (0.59,1.20)	70	0.70	0.83 (0.59,1.15)	0.67 (0.47,0.95)
Q4 (65)	99,752	76	0.91	1.05 (0.74,1.49)	1,069	0.85 (0.60,1.20)	82	0.78	0.92 (0.66,1.27)	1.04 (0.76,1.42)
Q5 (82)	100,026	63	0.67	0.80 (0.55,1.15)	1,713	0.88 (0.62,1.25)	90	0.75	0.89 (0.64,1.23)	0.83 (0.60,1.15)
p for trend			0.01	0.12		0.41		0.43	0.95	0.85
β-carotene										
Q1 (192)	98,210	69	1(Ref.)	1(Ref.)	2,439	1(Ref.)	58	1(Ref.)	1(Ref.)	1(Ref.)
Q2 (210)	99,137	73	0.81	0.88 (0.63,1.22)	3,666	1.00 (0.72,1.39)	75	1.05	1.15 (0.81,1.63)	0.85 (0.60,1.19)
Q3 (228)	99,549	66	0.69	0.77 (0.54,1.10)	4,782	0.74 (0.52,1.06)	83	1.06	1.23 (0.87,1.74)	1.03 (0.75,1.43)
Q4 (256)	99,826	76	0.71	0.83 (0.59,1.18)	6,257	0.89 (0.63,1.26)	87	1.00	1.19 (0.84,1.69)	0.84 (0.60,1.19)
Q5 (351)	100,195	61	0.58	0.69 (0.48,1.00)	9,409	0.86 (0.59,1.24)	75	0.85	1.00 (0.69,1.45)	0.93 (0.65,1.31)
p for trend			0.005	0.08		0.44		0.18	0.58	0.78
β-carotene from food										
Q1 (194)	90,917	66	1(Ref.)	1(Ref.)	2,273	1(Ref.)	55	1(Ref.)	1(Ref.)	1(Ref.)
Q2 (209)	90,665	53	0.64	0.69 (0.47,0.99)	3,362	0.95 (0.67,1.35)	55	0.82	0.90 (0.62,1.31)	0.92 (0.64,1.32)
Q3 (223)	90,047	71	0.76	0.85 (0.60,1.21)	4,315	0.86 (0.60,1.24)	65	0.86	0.99 (0.68,1.43)	0.90 (0.62,1.30)
Q4 (242)	88,893	72	0.69	0.81 (0.57,1.16)	5,502	1.06 (0.74,1.51)	83	1.02	1.20 (0.83,1.72)	1.13 (0.79,1.60)
Q5 (285)	86,289	55	0.49	0.60 (0.40,0.88)	7,758	0.75 (0.50,1.11)	82	0.89	1.04 (0.71,1.51)	0.94 (0.65,1.36)
p for trend			0.0008	0.04		0.24		0.93	0.51	0.93
Total carotene from food										
Q1 (246)	90,945	65	1(Ref.)	1(Ref.)	2,728	1(Ref.)	57	1(Ref.)	1(Ref.)	1(Ref.)
Q2 (265)	90,639	59	0.71	0.78 (0.54,1.12)	4,030	0.80 (0.56,1.15)	57	0.81	0.91 (0.62,1.32)	0.79 (0.55,1.13)
Q3 (282)	89,870	68	0.72	0.83 (0.58,1.18)	5,241	0.86 (0.61,1.23)	58	0.74	0.86 (0.59,1.25)	0.74 (0.52,1.06)
Q4 (307)	88,928	72	0.70	0.83 (0.58,1.18)	6,823	0.84 (0.59,1.20)	87	1.03	1.22 (0.85,1.74)	0.99 (0.71,1.40)
Q5 (364)	86,430	53	0.48	0.58 (0.39,0.87)	10,034	0.78 (0.53,1.13)	81	0.84	1.00 (0.68,1.44)	0.82 (0.57,1.18)
p for trend			0.0003	0.02		0.29		0.87	0.60	0.76

Supplementary Table 1.3. (Continued) Hazard ratios of AMD according to quintiles of predicted plasma carotenoid scores and calculated intakes in the HPFS

Total carotenoid index										
Q1 (9)	96,159	72	1(Ref.)	1(Ref.)	8	1(Ref.)	73	1(Ref.)	1(Ref.)	1(Ref.)
Q2 (12)	98,756	74	0.87	0.92 (0.66,1.28)	18	0.70 (0.49,1.00)	61	0.73	0.80 (0.57,1.12)	0.87 (0.62,1.21)
Q3 (15)	108,051	84	0.84	0.93 (0.67,1.28)	15	0.99 (0.72,1.36)	90	0.91	1.03 (0.75,1.42)	0.91 (0.66,1.26)
Q4 (18)	96,109	64	0.68	0.78 (0.55,1.11)	18	0.84 (0.60,1.18)	76	0.81	0.94 (0.67,1.32)	1.02 (0.74,1.40)
Q5 (21)	97,841	51	0.52	0.61 (0.42,0.89)	22	0.80 (0.56,1.12)	78	0.78	0.93 (0.66,1.31)	0.91 (0.66,1.27)
p for trend			0.0001	0.009		0.36		0.29	0.99	0.85

Multivariate models were adjusted for the same variables as the Supplementary Table 1.1 except postmenopausal status and hormone use and additionally adjusted for race (Caucasian v.s. non-Caucasian)

Supplementary Table 1.4. Association of first diagnosis of AMD with change in intakes of carotenoids and other nutrients

	All (n=422)			Intermediate (n=198)			Advanced (n=224)		
	Point Estimate	95% CI	P value	Point Estimate	95% CI	P value	Point Estimate	95% CI	P value
Lutein/zeaxanthin, µg									
β_2 (AMD)	204	(42, 366)	0.01	93	(-143, 330)	0.44	315	(93, 538)	0.006
β_3 (AMD*interval)	63	(0.83, 126)	0.05	38	(-48, 124)	0.39	93	(3, 183)	0.04
α-carotene, µg									
β_2 (AMD)	0.09	(-67.2, 67.4)	0.99	-21	(-114, 71)	0.66	18	(-76, 115)	0.69
β_3 (AMD*interval)	-0.23	(-28, 27.6)	0.99	3	(-43, 49)	0.9	-2.6	(-32, 27)	0.86
β-carotene , µg									
β_2 (AMD)	315.7	(-4.6, 636)	0.05	394	(-45, 832)	0.08	254	(-214, 722)	0.29
β_3 (AMD*interval)	6.7	(-122, 135)	0.92	-59	(-250, 132)	0.55	70	(-103, 244)	0.43
β-carotene from food, µg									
β_2 (AMD)	-23	(-235, 188)	0.83	-104	(-388, 181)	0.48	56	(-250, 362)	0.72
β_3 (AMD*interval)	73	(-18, 164)	0.11	59	(-78, 195)	0.4	92	(-26, 209)	0.13
β-cryptoxanthin, µg									
β_2 (AMD)	2.6	(-7.3, 12.5)	0.61	0.49	(-13, 14)	0.94	4.4	(-10, 19)	0.54
β_3 (AMD*interval)	-0.67	(-4.7, 3.3)	0.74	0.34	(-4.8, 5.5)	0.9	-1.6	(-7.8, 4.6)	0.61
Lycopene, µg									
β_2 (AMD)	72	(-253, 397)	0.67	167	(-266, 599)	0.45	-26	(-510, 459)	0.92
β_3 (AMD*interval)	42	(-87, 170)	0.53	76	(-105, 257)	0.41	2.4	(-180, 185)	0.98
Total fruits and vegetables, servings									
β_2 (AMD)	-0.17	(-0.33, 0.003)	0.05	0.17	(-0.40, 0.06)	0.15	-0.17	(-0.41, 0.08)	0.18
β_3 (AMD*interval)	0.08	(0.01, 0.14)	0.02	0.10	(0.02, 0.19)	0.02	0.05	(-0.05, 0.15)	0.32
DHA, mg									
β_2 (AMD)	-11	(-24, 3)	0.11	-12	(-28, 4)	0.13	-10	(-30, 11)	0.35
β_3 (AMD*interval)	9	(3, 15)	0.003	13	(6, 21)	<.001	4	(-5, 13)	0.35
ALA, mg									
β_2 (AMD)	-10	(-500, 30)	0.58	-26	(-8, 25)	0.32	3	(-56, 62)	0.92
β_3 (AMD*interval)	30	(12, 40)	<.001	32	(13, 51)	0.001	20	(-11, 50)	0.06

β_2= Change in calorie-adjusted carotenoid intake associated with a diagnosis of AMD during the middle of follow-up

β_3= Difference in the rate of change in calorie-adjusted carotenoid intake comparing AMD patients to non-AMD participants

Supplementary Table 2.1. Empirical prediction models for the measured plasma and erythrocyte EPA and DHA

	NHS					HPFS				
	Food (servings/d)	Cohort[‡], %	β	SE	p	Food (servings/d)	Cohort[§], %	β	SE	p
EPA										
Erythrocyte[¶]	Canned tuna	17.9	0.23	0.07	<.001	Canned tuna	10.2	0.37	0.08	<.001
(Mean ± SD)	Dark fish	38.2	1.27	0.16	<.001	Dark fish	43.4	1.33	0.11	<.001
(0.41 ± 0.16 %)	Other fish	20.0	0.23	0.08	0.003	Other fish	13.8	0.74	0.11	<.001
n=1,557	Shrimp and shellfish	8.2	0.69	0.18	<.001	n=1,364				
	Chicken w/ skin	12.1	0.17	0.07	0.01					
	R^2 = 0.09						R^2 = 0.19			
Plasma[¶]	Canned tuna	17.9	0.20	0.10	0.040	Plasma[¶]	Canned tuna	10.2	0.48	0.10	<.001
(Mean ± SD)	Dark fish	38.2	1.13	0.22	<.001	(Mean ± SD)	Dark fish	43.4	1.21	0.14	<.001
(0.45 ± 0.23%)	Other fish	20.0	0.25	0.11	0.02	(0.54 ± 0.37%)	Other fish	13.8	0.78	0.14	<.001
n=1,353	Shrimp and shellfish	8.2	0.66	0.25	0.009	n=1,321					
	R^2 = 0.05						R^2 = 0.14			
DHA										
Erythrocyte[¶]	Canned tuna	29.1	0.40	0.05	<.001	Erythrocyte[¶]	Canned tuna	19.8	0.42	0.06	<.001
(Mean ± SD)	Dark fish	22.3	1.23	0.11	<.001	(Mean ± SD)	Dark fish	29.5	0.90	0.08	<.001
(3.29 ± 0.98%)	Other fish	26.4	0.40	0.06	<.001	(3.32 ± 1.11%)	Other fish	23.2	0.60	0.08	<.001
n=1,966	Shrimp and shellfish	3.2	0.34	0.13	0.007	n=1,365	Chicken w/o skin	8.7	0.09	0.04	0.03
	R^2 = 0.17						R^2 = 0.25			
Plasma[¶]	Canned tuna	29.1	0.38	0.06	<.001	Plasma[¶]	Canned tuna	19.8	0.59	0.07	<.001
(Mean ± SD)	Dark fish	22.3	1.40	0.15	<.001	(Mean ± SD)	Dark fish	29.5	1.05	0.09	<.001
(1.42 ± 0.58%)	Other fish	26.4	0.46	0.07	<.001	(1.37 ± 0.60%)	Other fish	23.2	0.66	0.10	<.001
n=1,766	Shrimp and shellfish	3.2	0.46	0.17	0.007	n=1,319					
	R^2 = 0.14						R^2 = 0.24			

Supplementary Table 2.1. (Continued) Empirical prediction models for the measured plasma and erythrocyte EPA and DHA

‡ 1986-1990 average percent contribution to total intake in the NHS full cohort

§ 1990-1994 average percent contribution to total intake in the HPFS full cohort

¶ Mean level was expressed as the percentage of total fatty acids; biomarker concentrations were adjusted for age, BMI, pack-year of smoking, fasting status, case-control status, menopausal status and hormone use

¶ Total calories were forced into all models.

Supplementary Table 2.2. The Spearman correlations with measured EPA and DHA levels for dietary intake and predicted biomarker scores in the NHS and HPFS

			Predicted			Predicted
	n	Diet	Erythrocyte	n	Diet	Plasma
NHS						
EPA	1,557	0.28	0.30	1,353	0.19	0.21
DHA	1,966	0.44	0.45	1,766	0.39	0.40
HPFS						
EPA	1,364	0.50	0.46	1,321	0.42	0.38
DHA	1,365	0.56	0.53	1,319	0.53	0.51

P<.0001 for all the correlation coefficients

Supplementary Table 2.3. The multivariate hazard ratios of AMD according to dietary intake, predicted erythrocyte and plasma scores of EPA and DHA among non-users of fish oil supplements in the NHS and HPFS

		HR comparing the top to the bottom quintile			
		DHA	p trend	EPA	p trend
Intermediate AMD					
NHS	Diet	0.75 (0.61,0.92)	0.01	0.88 (0.71,1.08)	0.17
	Erythrocyte	0.79 (0.64,0.98)	0.04	0.79 (0.64,0.97)	0.04
	Plasma	0.76 (0.62,0.94)	0.04	0.79 (0.65,0.97)	0.06
HPFS	Diet	0.88 (0.62,1.25)	0.48	0.94 (0.66,1.33)	0.56
	Erythrocyte	0.70 (0.50,0.97)	0.02	0.76 (0.54,1.05)	0.06
	Plasma	0.74 (0.53,1.03)	0.03	0.72 (0.52,0.99)	0.03
Advanced AMD					
NHS	Diet	1.05 (0.84,1.31)	0.89	1.14 (0.91,1.43)	0.18
	Erythrocyte	1.09 (0.87,1.37)*	0.70	1.12 (0.90,1.40)*	0.22
	Plasma	1.17 (0.94,1.47)*	0.27	1.11 (0.89,1.38)*	0.25
HPFS	Diet	0.75 (0.51,1.09)	0.12	0.86 (0.58,1.25)	0.51
	Erythrocyte	0.54 (0.37,0.80)*	0.003	0.57 (0.39,0.85)*	0.008
	Plasma	0.55 (0.37,0.81)*	0.005	0.56 (0.38,0.83)*	0.003

*p for heterogeneity in the HR between the NHS and HPFS was < 0.05
Multivariate models were adjusted for the same variables as in the Table 2.2.

Supplementary Table 2.4. The multivariate hazard ratios of AMD stratified by the median intake of linoleic acid

		HR comparing Q5 to Q1				
		Low LA	p trend	High LA	p trend	p interaction
Intermediate AMD						
EPA	NHS	0.96 (0.73,1.25)	0.39	0.82 (0.64,1.07)	0.15	0.57
	HPFS	1.02 (0.64,1.61)	0.94	0.77 (0.49,1.21)	0.16	0.89
	pooled	0.99 (0.78,1.24)	0.61	0.80 (0.64,1.00)	0.04	0.51
DHA	NHS	0.78 (0.60,1.03)	0.09	0.76 (0.58,0.98)	0.04	0.38
	HPFS	1.00 (0.63,1.61)	0.73	0.72 (0.46,1.11)	0.17	0.27
	pooled	0.84 (0.67,1.07)	0.15	0.74 (0.59,0.92)	0.01	0.18
EPA + DHA	NHS	0.84 (0.64,1.09)	0.15	0.78 (0.61,1.01)	0.10	0.74
	HPFS	1.08 (0.68,1.71)	0.88	0.76 (0.49,1.19)	0.15	0.48
	pooled	0.91 (0.72,1.14)	0.26	0.77 (0.62,0.96)	0.02	0.54
EPA + DHA	NHS	0.90 (0.69,1.18)	0.53	0.80 (0.61,1.05)	0.07	0.59
(supplements users	HPFS	0.97 (0.59,1.59)	0.59	0.78 (0.49,1.24)	0.14	0.87
excluded)	pooled	0.93 (0.73,1.18)	0.44	0.79 (0.63,1.00)	0.02	0.42
Advanced AMD						
EPA	NHS	0.99 (0.74,1.33)	0.80	1.19 (0.89,1.58)	0.14	0.58
	HPFS	1.02 (0.67,1.57)	0.26	0.72 (0.43,1.20)	0.20	0.10
	pooled	1.01 (0.79,1.28)	0.54	1.04 (0.81,1.33)	0.98	0.99
DHA	NHS	1.00 (0.75,1.33)	0.55	1.10 (0.82,1.48)	0.58	0.73
	HPFS	0.90 (0.58,1.40)	0.82	0.74 (0.46,1.20)	0.14	0.32
	pooled	0.97 (0.76, 1.23)	0.77	0.98 (0.76, 1.26)	0.49	0.96
EPA + DHA	NHS	1.05 (0.78,1.41)	0.71	1.21 (0.91,1.61)	0.28	0.70
	HPFS	0.96 (0.61,1.50)	0.65	0.73 (0.44,1.21)	0.12	0.17
	pooled	1.03 (0.80,1.32)	0.97	1.06 (0.82,1.35)	0.72	0.98
EPA + DHA	NHS	0.99 (0.73,1.34)	0.64	1.19 (0.88,1.61)	0.21	0.60
(supplements users	HPFS	0.75 (0.46,1.20)	0.51	0.47 (0.25, 0.86)	0.007	0.07
excluded)	pooled	0.91 (0.71,1.18)	0.44	0.97 (0.74, 1.26)	0.31	0.93

The median intake of LA was 8.9 g/d in the NHS and 11g/d in the HPFS
Multivariate models were adjusted for the same variables as in the Table 2.2.

Supplementary Table 2.5. The multivariate hazard ratios of AMD stratified by the median age of onset of AMD cases

		HR comparing Q5 to Q1				
		age<73	p trend	age>=73	p trend	p interaction
Intermediate AMD						
EPA	NHS	0.80 (0.63,1.02)	0.10	0.99 (0.73,1.33)	0.52	0.16
	HPFS	1.09 (0.64,1.85)	0.87	0.75 (0.51,1.11)	0.21	0.34
	pooled	0.85 (0.68,1.05)	0.16	0.91 (0.72,1.15)	0.18	0.51
DHA	NHS	0.65 (0.51,0.83)	0.005	0.95 (0.71,1.27)	0.38	0.09
	HPFS	0.99 (0.60,1.64)	0.49	0.71 (0.48,1.06)	0.27	0.02
	pooled	0.70 (0.57,0.87)	0.006	0.87 (0.69,1.10)	0.16	0.64
EPA + DHA	NHS	0.68 (0.54,0.87)	0.10	1.00 (0.75,1.33)	0.72	0.25
	HPFS	1.09 (0.65,1.83)	0.54	0.77 (0.52,1.14)	0.27	0.12
	pooled	0.74 (0.60,0.92)	0.01	0.92 (0.73,1.16)	0.30	0.64
EPA + DHA	NHS	0.71 (0.56,0.91)	0.02	1.06 (0.79,1.43)	0.92	0.26
(supplements users	HPFS	1.02 (0.60,1.75)	0.59	0.72 (0.47,1.10)	0.11	0.07
excluded)	pooled	0.76 (0.61,0.95)	0.02	0.95 (0.74,1.20)	0.29	0.52
Advanced AMD						
EPA	NHS	0.92 (0.69,1.22)	0.49	1.17 (0.87,1.57)	0.20	0.02
	HPFS	0.84 (0.49,1.43)	0.82	0.95 (0.64,1.42)	0.98	0.54
	pooled	0.92 (0.72,1.19)	0.63	1.10 (0.87,1.39)	0.39	0.21
DHA	NHS	0.88 (0.65,1.17)	0.14	1.14 (0.86,1.53)	0.46	0.14
	HPFS	0.82 (0.49,1.37)	0.55	0.82 (0.55,1.23)	0.44	0.99
	pooled	0.88 (0.68,1.13)	0.18	1.04 (0.82,1.32)	0.99	0.31
EPA + DHA	NHS	0.92 (0.69,1.24)	0.21	1.24 (0.93,1.66)	0.20	0.11
	HPFS	0.80 (0.47,1.37)	0.63	0.89 (0.59,1.35)	0.50	0.83
	pooled	0.91 (0.71,1.18)	0.29	1.13 (0.89,1.43)	0.68	0.44
EPA + DHA	NHS	0.99 (0.73,1.33)	0.49	1.09 (0.80,1.48)	0.40	0.07
(supplements users	HPFS	0.54 (0.29,0.99)	0.09	0.67 (0.42,1.06)	0.05	0.68
excluded)	pooled	0.90 (0.69,1.17)	0.16	0.94 (0.73,1.22)	0.44	0.47

Multivariate models were adjusted for the same variables as in the Table2.2

Supplementary Table 3.1. The associations between intake of ALA and AMD stratified by smoking status

	No. of cases	Q1	Q2	Q3	Q4	Q5	p trend	p interaction
Intermediate AMD								
NHS								
Never smokers	528	1 (ref)	1.30 (0.97,1.75)	1.23 (0.90,1.68)	1.40 (1.02,1.93)	1.69 (1.20,2.37)	0.003	0.08
Ever smokers	681	1 (ref)	1.15 (0.90,1.48)	0.93 (0.71,1.23)	1.37 (1.05,1.79)	1.10 (0.81,1.48)	0.43	
HPFS								
Never smokers	150	1 (ref)	1.38 (0.80,2.37)	1.21 (0.68,2.16)	1.52 (0.86,2.69)	1.61 (0.88,2.93)	0.14	
Ever smokers	230	1 (ref)	0.89 (0.59,1.35)	0.78 (0.51,1.21)	0.78 (0.50,1.21)	0.98 (0.63,1.54)	0.92	0.43
Pooled								
Never smokers	*678*	*1 (ref)*	*1.31 (1.01,1.70)*	*1.22 (0.92,1.60)*	*1.42 (1.07,1.88)*	*1.66 (1.23,2.22)*	*0.001*	
Ever smokers	*911*	*1 (ref)*	*1.08 (0.87,1.34)*	*0.89 (0.71,1.12)*	*1.19 (0.95,1.49)*	*1.07 (0.83,1.37)*	*0.51*	*0.10*
Advanced AMD								
NHS								
Never smokers	379	1 (ref)	1.07 (0.77,1.49)	1.01 (0.71,1.43)	0.78 (0.53,1.14)	1.04 (0.7,1.55)	0.85	
Ever smokers	631	1 (ref)	1.05 (0.81,1.37)	1.23 (0.94,1.61)	1.18 (0.89,1.57)	1.09 (0.8,1.49)	0.57	0.39
HPFS								
Never smokers	126	1 (ref)	0.91 (0.52,1.59)	0.80 (0.45,1.44)	0.83 (0.45,1.51)	0.94 (0.50,1.76)	0.83	
Ever smokers	220	1 (ref)	1.20 (0.78,1.85)	1.08 (0.68,1.69)	0.93 (0.58,1.49)	1.06 (0.65,1.73)	0.87	0.95
Pooled								
Never smokers	*505*	*1 (ref)*	*1.03 (0.78,1.37)*	*0.95 (0.71,1.28)*	*0.79 (0.57,1.09)*	*1.02 (0.73,1.42)*	*0.78*	
Ever smokers	*851*	*1 (ref)*	*1.10 (0.87,1.37)*	*1.19 (0.94,1.49)*	*1.12 (0.88,1.42)*	*1.09 (0.84,1.41)*	*0.66*	*0.57*

Multivariate model included: age (continuous), race (Caucasians or not), BMI (<18.5, 18.5-23, 23-25, 25-30, 30-35, ≥ 35 kg/m2), physical activity (<3, 3-8.9, 9-17.9, 18-26.9, ≥27 MET-h/wk), current aspirin use (≥1 tablets/wk or none), history of hypertension, history of hypercholesterolemia, total calories, DHA and LA (all in quintiles). In the NHS, models were additionally adjusted for postmenopausal status and menopausal hormone use (never, current and past)

Supplementary Table 3.2. The associations between intake of ALA and AMD stratified by age

	No. of cases	Q1	Q2	Q3	Q4	Q5	p trend	p interaction
Intermediate AMD								
NHS								
Age<73 years old	710	1 (ref)	1.07 (0.83,1.38)	1.00 (0.77,1.31)	1.28 (0.98,1.67)	1.25 (0.94,1.66)	0.08	0.48
Age≥73 years old	499	1 (ref)	1.43 (1.06,1.91)	1.11 (0.80,1.54)	1.52 (1.10,2.10)	1.39 (0.97,1.98)	0.11	
HPFS								
Age<73 years old	154	1 (ref)	1.45 (0.83,2.51)	1.24 (0.69,2.23)	1.30 (0.72,2.35)	1.83 (1.02,3.3)	0.07	0.2
Age≥73 years old	226	1 (ref)	0.86 (0.57,1.30)	0.78 (0.51,1.20)	0.90 (0.59,1.38)	0.90 (0.56,1.43)	0.76	
Pooled								
Age<73 years old	*864*	*1 (ref)*	*1.14 (0.91,1.43)*	*1.05 (0.82,1.33)*	*1.30 (1.02,1.65)*	*1.37 (1.06,1.76)*	*0.009*	*0.49*
Age≥73 years old	*725*	*1 (ref)*	*1.21 (0.95,1.53)*	*0.98 (0.76,1.27)*	*1.27 (0.98,1.64)*	*1.20 (0.91,1.59)*	*0.27*	
Advanced AMD								
NHS								
Age<73 years old	494	1 (ref)	1.21 (0.90,1.63)	1.25 (0.92,1.70)	1.00 (0.72,1.39)	1.06 (0.75,1.50)	0.82	
Age≥73 years old	516	1 (ref)	0.95 (0.71,1.26)	1.03 (0.77,1.39)	1.03 (0.75,1.40)	1.08 (0.77,1.52)	0.56	0.76
HPFS								
Age<73 years old	133	1 (ref)	2.00 (1.09,3.68)	1.98 (1.06,3.70)	1.63 (0.84,3.16)	1.81 (0.90,3.62)	0.32	
Age≥73 years old	213	1 (ref)	0.80 (0.53,1.22)	0.68 (0.43,1.06)	0.68 (0.43,1.07)	0.76 (0.48,1.22)	0.27	0.09
Pooled								
Age<73 years old	*627*	*1 (ref)*	*1.35 (1.03,1.76)*	*1.40 (1.07,1.85)*	*1.15 (0.86,1.54)*	*1.27 (0.93,1.72)*	*0.44*	
Age≥73 years old	*729*	*1 (ref)*	*0.91 (0.71,1.15)*	*0.91 (0.71,1.16)*	*0.90 (0.70,1.17)*	*0.96 (0.73,1.27)*	*0.83*	*0.27*

The median age of AMD onset was 73 years old.

Multivariate model included: age (continuous), race (Caucasians or not), BMI (<18.5, 18.5-23, 23-25, 25-30, 30-35, ≥ 35 kg/m2), pack-years of smoking (never, 1-9, 10-24, 25-44, 45-64, ≥65y), physical activity (<3, 3-8.9, 9-17.9, 18-26.9, ≥27 MET-h/wk), current aspirin use (≥1 tablets/wk or none), history of hypertension, history of hypercholesterolemia, total calories, DHA and LA (all in quintiles). In the NHS, models were additionally adjusted for postmenopausal status and menopausal hormone use (never, current and past)

Supplementary Table 3.3. Stepwise regression models for erythrocyte *cis* and *trans* ALA

cis ALA[¶] N=395			*trans* ALA-B[¶] N=392			*trans* ALA C+ D[¶] N=391		
Mean ± SD, %: 0.13 ± 0.04			Mean ± SD, %: 0.01 ± 0.004			Mean ± SD, %: 0.03 ± 0.01		
Food	β	p	Food	β	p	Food	β	p
Mayonnaise	0.31	<.0001	Mayonnaise	0.31	<.0001	Beef	-0.27	0.004
Broccoli	0.20	0.03	Pie (store-made)	1.35	0.01	Butter	-0.07	0.007
			Peanuts	-0.21	0.02	Ice cream	-0.22	0.02
			Brownie	0.86	0.03	Muffin	0.28	0.03
			Oil & vinegar dressing	0.12	0.03	Chicken w/ skin	-0.25	0.04
R^2=0.07			R^2=0.10			R^2=0.08		

[¶]Because of missing data or outliers, sample size for each model was not the same

Supplementary Table 3.4. Pearson correlations between erythrocyte *cis* and *trans* ALA

	cis ALA	*trans* ALA-B	*trans* ALA-C+ D
cis ALA	1		
trans ALA-B	0.65 (n=392)	1	
trans ALA-C+ D	0.17 (n=391)	0.20 (n=388)	1

P<.001 for all the correlation coefficients.

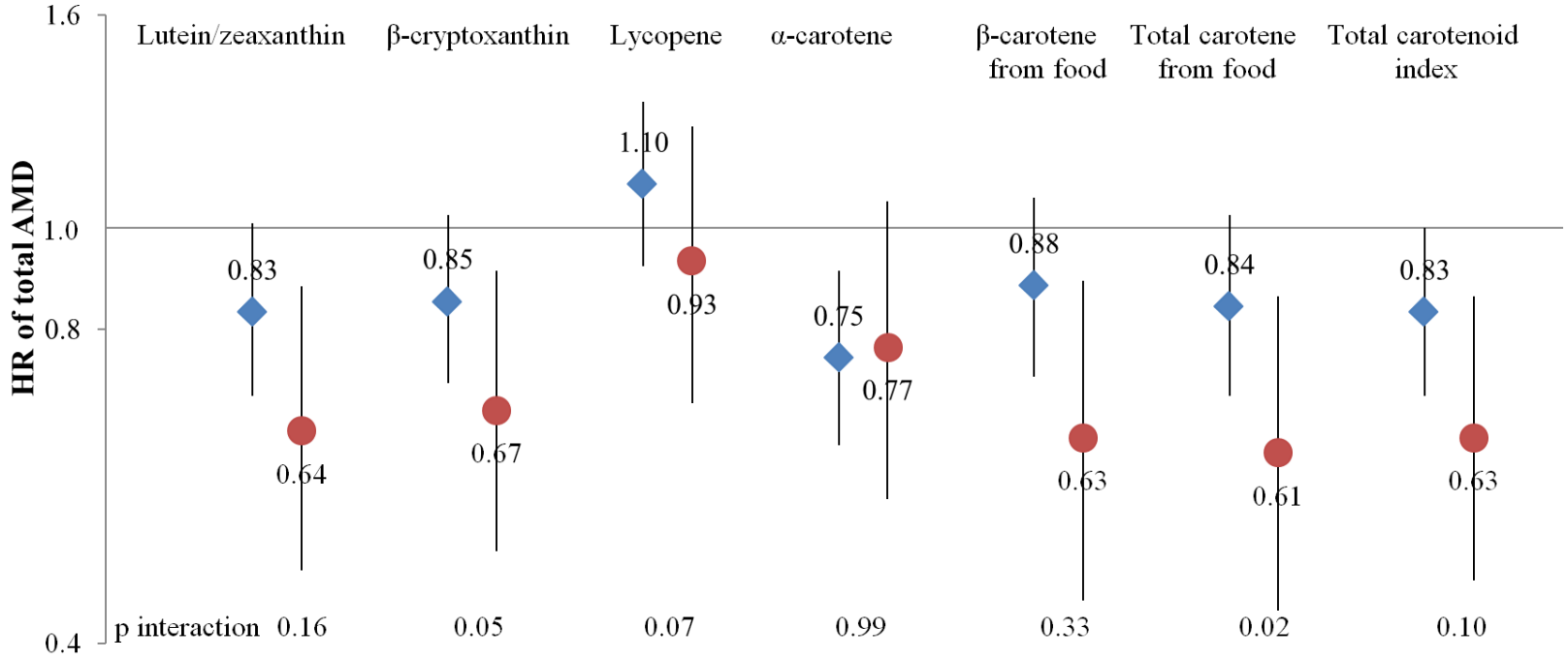
Supplementary Table 3.5. Stepwise regression models for plasma and erythrocyte *cis* ALA among previously measured blood samples

	NHS[‡]					HPFS[§]				
	Food	Cohort[φ], %	β	SE	p	Food	Cohort[φ], %	β	SE	p
ALA										
Plasma[¶]	Mayonnaise	15.4	0.11	0.03	<.0001	Mayonnaise	11.32	0.16	0.04	<.0001
(Mean ± SD, %)	Iceberg lettuce	12.4	0.05	0.02	0.005	Beef, pork or lamb	4.63	-0.18	0.07	0.009
(0.49 ± 0.16)	Beef, pork or lamb	5.4	-0.11	0.04	0.002	American cheese	2.79	-0.09	0.03	0.002
n=1,810	American cheese	3.7	-0.05	0.02	0.009	Dark bread	0.75	0.02	0.01	0.03
	Oil & vinegar dressing	2.0	0.06	0.02	0.009	Chicken w/o skin	2.52	0.10	0.05	0.04
	R^2 = 0.03					R^2 = 0.03				
Erythrocyte[¶]	Mayonnaise	15.4	0.14	0.03	<.0001	Erythrocyte[¶]	Mayonnaise	11.32	0.12	0.04	0.004
(Mean ± SD, %)	Beef, pork or lamb	5.4	-0.08	0.04	0.04	Beef, pork or lamb	4.63	-0.22	0.07	0.002
(0.15 ± 0.05)	Oil & vinegar dressing	2.0	0.09	0.03	0.0004	(0.17 ± 0.04)	American cheese	2.79	-0.09	0.03	0.004
n=2,033						n=1,414	Processed meat	1.82	-0.09	0.04	0.02
							Pizza	1.78	-0.27	0.13	0.04
	R^2 = 0.02					R^2 = 0.03				

[‡] 1986-1990 average percent contribution to total intake in the NHS full cohort
[§] 1990-1994 average percent contribution to total intake in the HPFS full cohort
[φ] % contribution to the calculated intake in the cohort
[¶] Mean level was expressed as the percentage of total fatty acids; biomarker concentrations were adjusted for age, BMI, pack-year of smoking, fasting status, case-control status, menopausal status and hormone therapy
[¶] Total calories were forced into all models

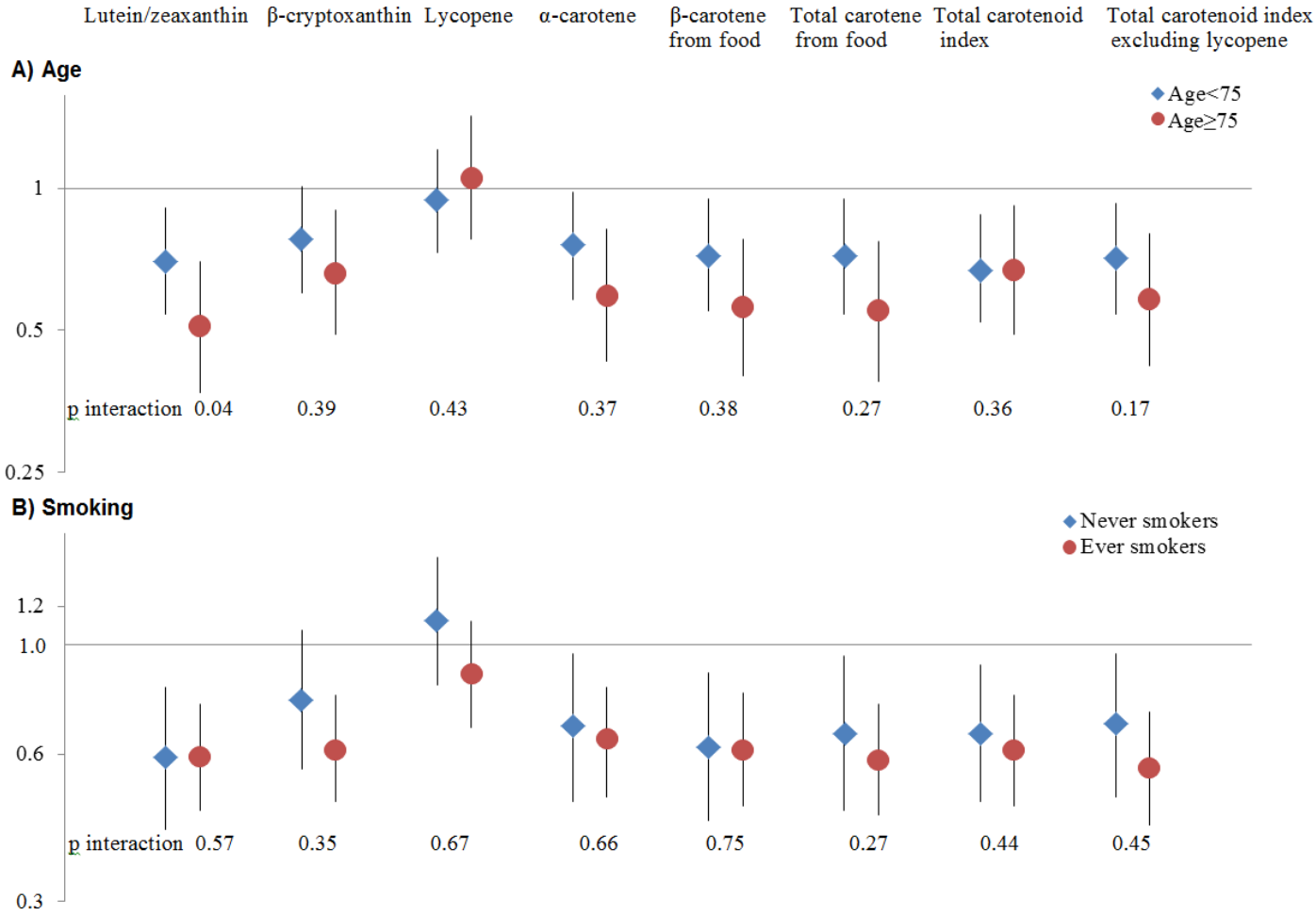
Supplemental Figures

Supplementary Figure 1.1. Predicted plasma carotenoid scores and HRs of total AMD according to current menopausal hormone use status among postmenopausal women



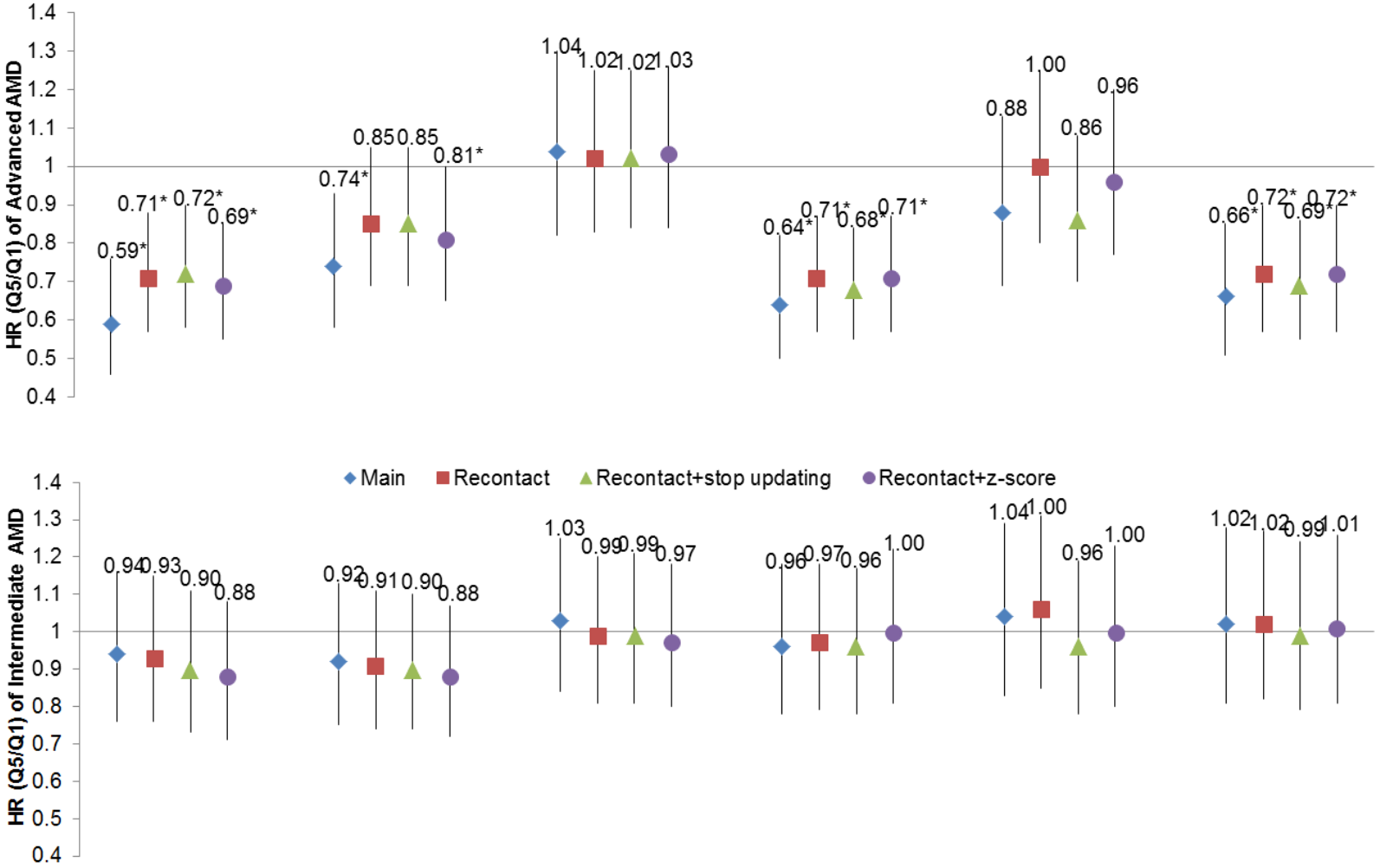
There were 1,221 cases in non-current users group and 413 cases in current users group
All the HRs were comparing the fifth to the bottom quintile
Multivariate models were adjusted for: age (continuous), BMI (≥30 kg/m^2), pack-years of smoking (never, 1-9, 10-24, 25-44, 45-64, ≥65y), physical activity (<3, 3-8.9, 9-17.9, 18-26.9, ≥27 MET-h/wk), current aspirin use (≥1 tablets/wk or none), aHEI (excluding fruits and vegetables)

Supplementary Figure 1.2. Predicted plasma carotenoid scores and pooled HRs of advanced AMD according to age and smoking status



A) Multivariate models were adjusted for the same variables as in Supplementary Figure 1.1 and additionally adjusted for race in HPFS; 651 cases in age<75 group (477 from NHS and 174 cases from HPFS); 467 cases in age≥75 group (296 from NHS and 171 cases from HPFS).
B) Multivariate models were adjusted for the same variables as in A) except pack-year of smoking; 425 cases in never smokers group (289 from NHS and 136 cases from HPFS); 693 cases in ever smokers group (484 from NHS and 209 cases from HPFS)

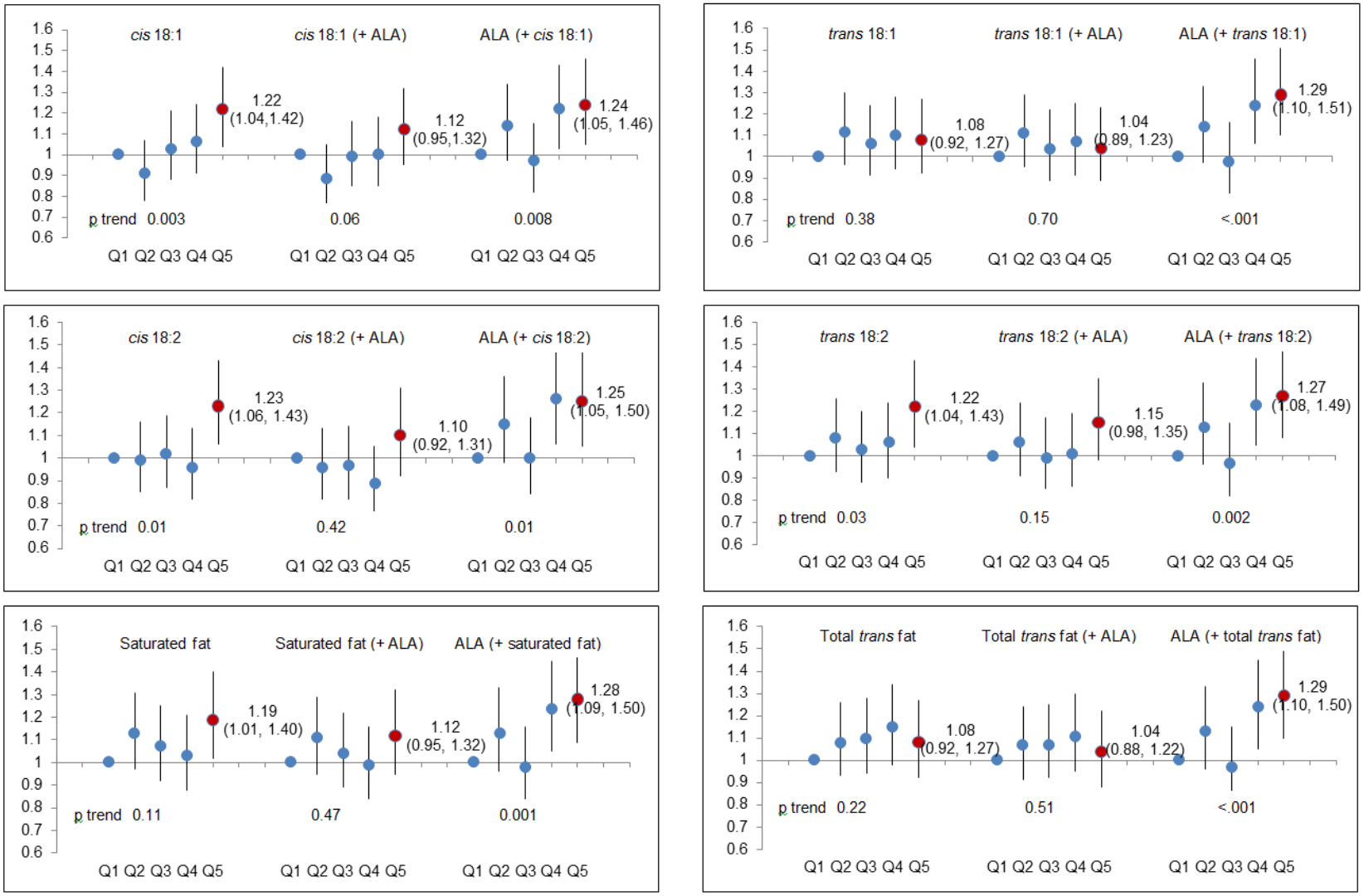
Supplementary Figure 1.3. Hazard ratios comparing extreme quintiles for the associations between predicted plasma carotenoid scores and AMD in the NHS



*: p for trend <0.05

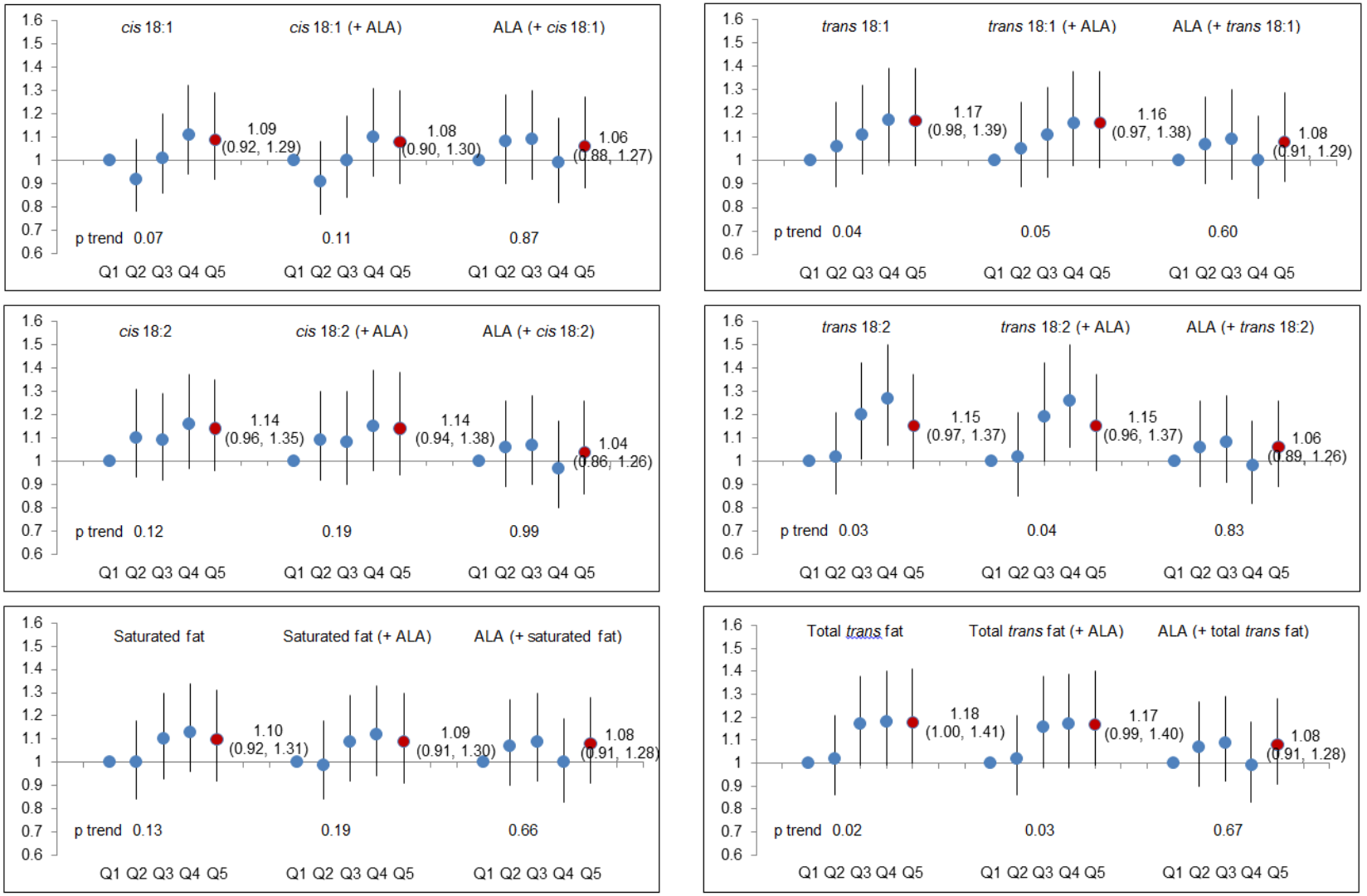
Multivariate models were adjusted for the same variables as in the Supplementary Figure 1.1

Supplementary Figure 3.1. The associations between different types of fatty acids and intermediate AMD



All the HRs were adjusted for the same variables as in the Figure 3.2

Supplementary Figure 3.2. The associations between different types of fatty acids and advanced AMD



All the HRs were adjusted for the same variables as in the Figure 3.2